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Volume 59 OCTOBER, 1956 Number 4

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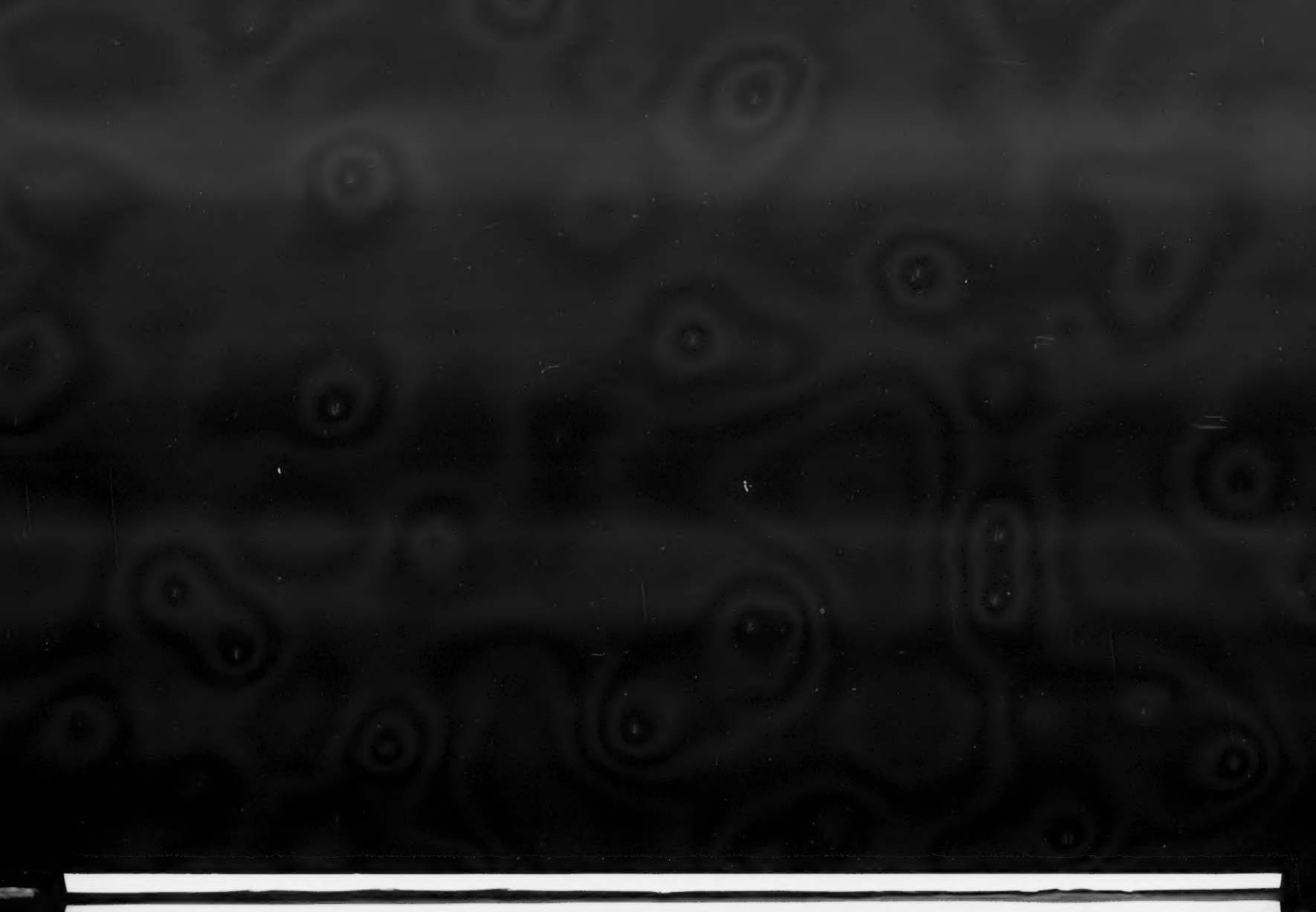

ENDOCRINOLOGY

VOLUME 59 OCTOBER, 1956 NUMBER 4

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THE EFFECT OF ETHYL ALCOHOL ON ADRENAL CORTICAL ACTIVITY IN MICE[1,2]

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IT HAS been suggested that a hypofunction of the pituitary-adrenal system may be an important factor in the etiology of chronic alcoholism (1–5). Accordingly, treatment of alcoholic states using ACTH and/or adrenocortical steroid hormones has been attempted (3, 6–11). Although no animal experiments have been reported which would relate chronic alcoholism to a pituitary-adrenal dysfunction, depletion of the adrenal ascorbic acid and cholesterol content was found following acute alcohol intoxication in intact rats (4, 12), and Guinea pigs (13, 14, 15).

In this study, the "thymo-regressive response" (16) has been utilized to make qualitative and quantitative evaluations of the increase in adrenocortical activity associated with acute alcoholic intoxication. This was done by determining the amount of thymic atrophy which occurred in a group of intact mice given an intoxicating dose of ethyl alcohol and comparing this with the thymo-regressive responses of groups of adrenalectomized mice receiving a similar dose of alcohol concurrent with one of a series of graded doses of Compound F acetate (17-hydroxy-corticosterone acetate), and also with the thymic atrophy which occurred in adrenalectomized animals treated only with the graded doses of the hormone.

EXPERIMENTAL

Two experiments were performed using two strains of mice. In the first experiment, 56 virgin female, 10-week-old, CBA mice were used. The animals were maintained under

Received November 17, 1955.

[1] Part of the study was completed at the Medical College of Virgnia.

[2] Supported by a grant from the Utah State Board on Alcoholism and grant number C-2607 from the United States Public Health Service.

optimal laboratory conditions at all times and their birth dates were accurate to within 4 days. Forty-nine animals were adrenalectomized, at which time their body weights were recorded; 24 hours postoperatively three groups of six animals each were given intraperitoneal injections of alcohol (3000 mg./kg.), and each group was then given a different dose of Compound F (25, 50 or 250 μg./mouse). Four groups (4 animals in each group) of adrenalectomized animals were treated only with one of 4 graded doses of the hormone (5, 25, 50, and 250 μg./mouse). Seven intact non-treated and 5 intact saline-injected (0.2 cc.) and 6 intact alcohol injected (3000 mg./kg.) animals served as controls. The injections were repeated 32 hours postoperatively and 8 hours after the last injection all animals were sacrificed by ether inhalation. The thymi were carefully dissected and their wet weights determined to the nearest .5 mg. The mean relative weights (mg. /100 gm. initial body weights) of the thymi were calculated and plotted against the log-dose hormone and the resulting response curves of the thymi of the experimental animals were mathematically fitted by the method of least squares (16, 17, 18).

In the second experiment, 49 female Swiss-Webster mice were used. These animals were obtained from commercial sources and there were greater variations in their ages and body weights. The experimental procedures were identical, with but a slight variation in the number of animals in the groups and in the dosages of hormone used.

The alcohol used in these experiments was Ethyl Alcohol U.S.P. diluted with .85% saline. The required doses of the suspended hormone (Hydrocortisone, Merck) were diluted in a constant volume of 0.2 cc. pyrogen free saline. The same volume of saline was used in the saline-injected controls.

RESULTS

The results of the first experiment, using adrenalectomized CBA mice, are presented in Tables 1 and 2 in which the observed mean relative thymic weights of the groups receiving only the graded doses of hormone and of those receiving the graded doses of hormone in addition to a uniform dose of alcohol are tabulated in order of increasing magnitude of the administered hormone dose. The calculated relative weights as obtained by math-

TABLE 1. MEAN RELATIVE WEIGHTS* OF THYMI OF ADRENALECTOMIZED CBA MICE GIVEN ALCOHOL† AND TREATED WITH GRADED DOSES OF COMPOUND F OR TREATED WITH COMPOUND F ONLY

Dose of hormone in μg.	Compound F only		Alcohol and compound F	
	Observed weight	Calculated weight‡	Observed weight	Calculated weight‡
5	107.97	111.09		
25	90.12	85.43	94.7	88.43
50	75.21	74.38	66.7	75.69
250	46.29	48.72	48.8	46.10
S.E. b		−36.71		−42.33
S.E. b		±1.19		±3.88

Intact controls	*Non-treated*		*Saline-treated*		*Alcohol-treated*
	107.8±5.06	(P<.001)	87.2±11.9	(P<.01)	52.5±7.2
	←———		(P<.001)		———→

* Mg./100 gm. initial body weight.
† 3000 mg./kg.
‡ Fitted according to $Y=a+bX$.

TABLE 2. MEAN RELATIVE WEIGHTS* OF THYMI OF ADRENALECTOMIZED SWISS-WEBSTER MICE GIVEN ALCOHOL† AND TREATED WITH GRADED DOSES OF COMPOUND F OR TREATED WITH COMPOUND F ONLY

Dose of hormone in μg.	Compound F only		Alcohol and compound F	
	Observed weight	Calculated weight‡	Observed weight	Calculated weight‡
10	250.9	245.0	247.2	240.1
25			219.6	225.2
50	208.7	214.2	214.1	213.9
500	175.3	170.2	175.8	176.3
b		−44.06		−37.59
S.E. *b*		±2.31		±5.62

Intact controls	*Non-treated*		*Alcohol-treated*
Intact controls	245.19 ± 27.9	(P < .1)	158.22 ± 27.5

* Mg./100 gm. initial body weight.
† 3000 mg./kg.
‡ Fitted according to $Y = a + bX$.

ematically fitting rectilinear regression lines from the observed weights are also presented. In both experiments, at any dose of hormone, the differences between the observed and calculated thymic weights are very small; thus demonstrating the closeness of fit of the calculated regression lines. The equation of these lines is $y = a - bx$, where y = the response expressed as the weight of the thymus in mg./100 gm. initial body weight; x = log-dose hormone; a = the y intercept; and b = the slope of the line. The slope values b with their standard errors S. E. b are also presented in the table. Negative slopes indicate involution; thus the calculated slope values represent the amount of decrease in relative weight in the thymus which occurred with every tenfold increase in dose of administered hormone. The fitted regression lines, as depicted in Figures 1 and 2, illustrate the significant log-dose response relationship between the decrease in thymic weight and the increase in dose of administered hormone. Alcohol treatment did not alter this relationship in any visible manner, thus the slopes and positions of the two regression lines in each experiment were virtually identical. In these figures, the shaded bars represent the mean relative thymic weights and their standard errors as obtained in the control groups. In the first experiment, the relative thymic weights of the intact animals injected with either alcohol or saline were significantly smaller ($P < .001$) than those of the intact non-treated controls. Also, those of the alcohol-treated animals were significantly smaller ($P < .01$) than of those treated with saline only.

The larger observed and calculated thymic weights of the animals in the second experiment would place their regression lines far to the right of those in experiment one. (Compare Tables 1 and 2.) These differences may be ascribed to the larger thymi of the Swiss-Webster strain of mice and were probably due to age and strain differences. The greater variation in

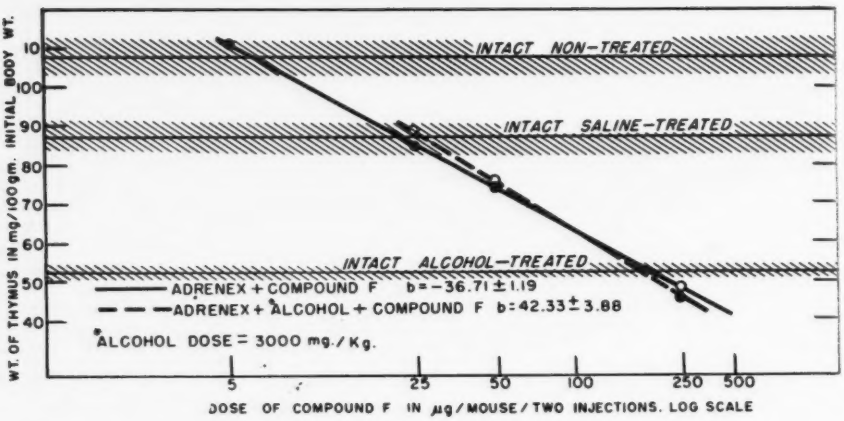
INTACT NON-TREATED
INTACT SALINE-TREATED
INTACT ALCOHOL-TREATED
ADRENEX + COMPOUND F b=-36.71±1.19
ADRENEX + ALCOHOL + COMPOUND F b=42.33±3.88
ALCOHOL DOSE = 3000 mg./Kg.
WT. OF THYMUS IN mg/100gm. INITIAL BODY WT.
DOSE OF COMPOUND F IN µg/MOUSE/TWO INJECTIONS. LOG SCALE

FIG. 1. Log dose-response relationships between the relative weight of the thymus and graded doses of Compound F (hydrocortisone) in adrenalectomized CBA mice treated and non-treated with two intoxicating doses of ethyl alcohol. Shaded bands represent mean and standard errors of mean thymic weights of control groups.

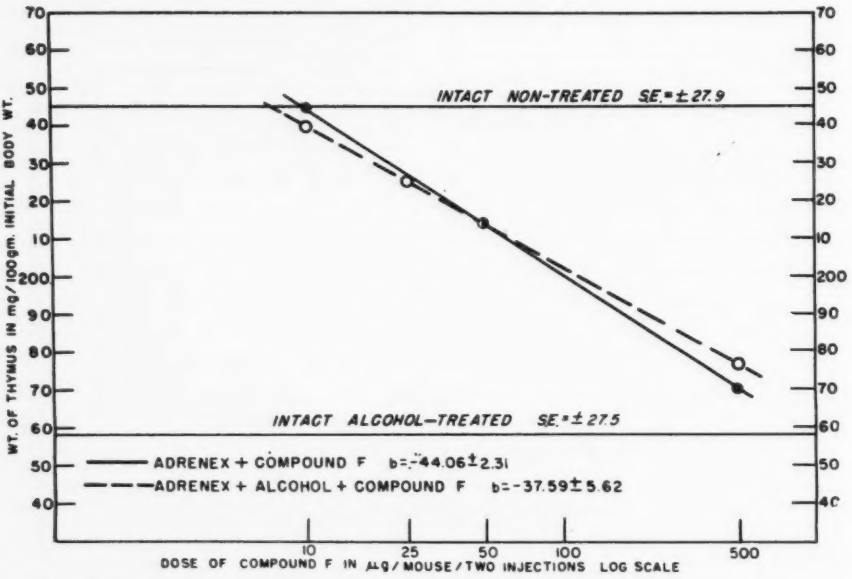
INTACT NON-TREATED SE.=±27.9
INTACT ALCOHOL-TREATED SE.=±27.5
ADRENEX + COMPOUND F b=-44.06±2.31
ADRENEX + ALCOHOL + COMPOUND F b=-37.59±5.62
WT. OF THYMUS IN mg/100gm. INITIAL BODY WT.
DOSE OF COMPOUND F IN µg/MOUSE/TWO INJECTIONS LOG SCALE

FIG. 2. Log dose-response relationships between the relative weight of the thymus and graded doses of Compound F in adrenalectomized Swiss-Webster mice treated and non-treated with two intoxicating doses of ethyl alcohol. Horizontal lines represent mean thymic weights of control groups.

the size of the thymi of the animals in this group led to larger standard errors of the mean slopes of the regression lines and of the mean relative thymic weights of the control groups. This was probably due to a greater variation in the age of these animals and to the possible presence of non-specific stress factors.

DISCUSSION

The cholesterol and ascorbic acid depleting effects of alcohol observed in intact but not hypophysectomized rats (4, 12) and Guinea pigs (13, 14, 15) indicate that an acute intoxicating dose of alcohol increases the activity of the adrenal cortex by way of the pituitary. However, these methods of evaluating adrenocortical activity do not give information concerning the types, amounts or biological activities of the substances being secreted (19). The thymo-regressive response, as utilized in this study, is a reliable indirect index of increased adrenocortical steroid hormone secretion, as this effect is specifically due to the activity of certain of those corticoids which are known to be essential for life and for maintaining the resistance of animals to the damaging effects of noxious stimuli (16, 20, 21).

The significant acute involution of the thymus which occurred in the intact animals given an intoxicating dose of alcohol may be ascribed to the effect of alcohol, *per se*, to its diluent, or indirectly to an increased secretion of adrenocortical hormones. Experiments in which alcohol or saline have been administered to adrenalectomized animals have not evoked this response in the thymus or lymph nodes (22). Thus, the possibility of a direct action of these substances on lymphatic tissue is excluded. Acute involution of the thymus is characteristically observed following exposure of intact animals to stressful stimuli (23) and has been attributed to the enhanced secretion of adrenocortical steroid hormones (20, 24). The thymic involution, observed following acute alcohol intoxication, may thus be ascribed to an increased adrenocortical activity leading to an increase in the level of active adrenocortical steroid hormones; presumably those with an oxy or hydroxy at C-11, since these are the only adrenocortical steroids which have so far been shown capable of inducing a thymo-regressive effect (16).

The differences in the relative positions of the response lines as obtained in experiments one and two may be attributed to the larger size of the thymi of the younger animals in the second experiment. The amount of decrease in thymic weight, per unit of hormone used, however, was virtually the same in both experiments. Thus, the thymo-regressive response appears to be independent of the initial size of the thymus.

The mechanism by which the adrenocortical hormones induce their thymo-regressive effect has been discussed elsewhere (16, 20). Differences in the slopes of the response lines obtained by different thymo-regressive substances are presumably due to differences in the modes of action of such

substances on the cell types of the thymus. In this study, the parallelism of the response lines of the hormone-treated animals, whether or not treated with alcohol, indicates that the alcohol did not in any visible manner alter the mechanism by which the hormone induced thymic involution. Similarly, the fact that in each experiment the regression lines of the alcohol-treated groups were identical in position with those of the non-alcohol-treated animals demonstrates that alcohol had neither a synergistic nor antagonistic effect on the thymo-regressive properties of Compound F. Synergism would have caused a leftward shift and antagonism a rightward shift of the response lines.

An estimate of the relative increase in adrenocortical activity which occurred in the intact alcohol-treated animals may be obtained by determining the increase in dose of Compound F which would be required to induce a thymic atrophy in adrenalectomized animals comparable to that observed in the intact alcohol-treated group. This was done graphically by noting the points of intersection of the thymo-regressive response lines as obtained using Compound F alone with the horizontal lines representing the thymic weights of the intact non-stressed and intact alcohol-stressed groups. In both experiments, the normal thymic-maintenance dose was found to be <10 μg./day which compares favorably with values previously reported (16). Thymic involution comparable to that obtained in the alcohol-treated animals was obtained with <250 >150 μg. and <500 μg. of Compound F in the CBA and Swiss-Webster strains of mice respectively. It may thus be stated that the administration of alcohol induced a twenty-five to fifty fold increase in adrenocortical activity, using the thymus maintenance dose of Compound F as a standard of reference.

It is well known that adrenalectomized animals are less resistant to many types of noxious stimuli and that "stress-resistance" of adrenalectomized animals may be restored by the administration of adrenocortical hormones (21, 25, 26). Accordingly, it has also been found that adrenalectomized non-hormone treated mice do not survive when treated with doses of alcohol one third as high as the one used in the present experiments (22). The adrenalectomized animals used in these experiments, however, were able to withstand a high dose of alcohol, when treated even with the lowest dose of Compound F. It would, therefore, appear that Compound F might be beneficial in increasing the resistance to the toxic effects of alcohol in the event of a concomitant adrenal cortical deficiency.

SUMMARY

Acute alcohol intoxication induced a decrease in the thymic weights of intact mice which was comparable to that observed in adrenalectomized animals treated with 250 to 500 μg. of Compound F (17 hydroxy-corticosterone). These doses are twenty-five to fifty times greater than the dose required to maintain thymic weights of adrenalectomized animals at the

level of intact non-stressed controls. Thus, using the thymus maintenance dose of Compound F as a standard of reference, there was a twenty-five to fifty fold increase in corticoid activity associated with the alcohol treatment. Analysis of the thymo-regressive response lines showed no evidence that alcohol induced synergistic or antagonistic effects, or alterations in the mode of action of the hormone on the thymus.

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THE LOCALIZATION OF GLYCOPROTEIN HORMONES IN THE ANTERIOR PITUITARY GLANDS OF RATS INVESTIGATED BY DIFFERENTIAL PROTEIN SOLUBILITIES, HISTOLOGICAL STAINS AND BIO-ASSAYS[1,2]

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ONE of the physical properties of proteins, that of differential solubility, is not usually taken advantage of when the routine methods of fixation are used prior to histological or histochemical staining procedures. However, it should be possible to expose a tissue containing a mixture of proteins to a reagent in which some of the proteins were denatured and rendered insoluble, while other soluble proteins were not denatured and were extracted. Because of the reversibility of denaturation with some reagents and because solvents are employed in the preparation of tissue sections, it is possible to use a second fixative containing a reagent that will combine with the proteins which were not extracted, precipitating them irreversibly so that they can be subsequently stained. In this manner, it should be possible to extract certain proteins from a mixture of proteins in a tissue, following which comparison of stained sections of the extracted tissue with sections of the same tissue in which all the proteins had been precipitated, would reveal the sites of the extracted proteins. After this procedure, which reduced the number of proteins, it should be possible to characterize the remainder more exactly by various staining reactions.

In the present work, the above hypothesis was applied to the anterior pituitary gland with the view of exploring methods by which the various hormones of the gland could be differentiated and assigned to the several cell types currently recognized. For convenience, as well as simplicity, we have divided the cells of the anterior pituitary gland into three types,

Received March 9, 1956.

[1] This work was supported in part by a grant (A-452(C2)) from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Department of Health, Education and Welfare, in part by an Institutional grant to Harvard University from the American Cancer Society and in part by a grant from the Massachusetts Division of the American Cancer Society.

[2] The technical assistance of Mrs. Françoise Puvrez Hall is gratefully acknowledged.

[3] Fellow of the American Cancer Society, upon recommendation of the Committee on Growth, National Research Council, 1952–1955. U.S.P.H. Special Research Fellow 1955–1956.

namely: (a) cells that stain with the periodic acid-Schiff (PAS) method ("basophiles"); (b) cells that stain with either Orange G (1) or the sulfhydryl and disulfide method of Barrnett and Seligman (2, 3, 4) ("acidophiles"); and (c) cells which contain little or no stainable material ("chromophobes").

In this paper, as in the preliminary reports (5, 6), we will deal largely with the cells which are PAS-positive and diastase-fast and which stain with PAS presumably because they contain the glycoprotein hormones namely, the follicle-stimulating hormone (FSH), the luteinizing hormone (LH) (gonadotropins), and the thyroid-stimulating hormone (TSH). The various solvents used to dissolve or precipitate these hormones differentially were suggested by the work of Fevold, Lee, Hisaw and Cohn (7), Fevold (8), Fraenkel-Conrat, Simpson and Evans (9), Li, Simpson and Evans (10, 11), Li and Evans (12) and Li (13). The pituitary glands of donor rats treated with various solvents were assayed for their hormone content by administering them to immature hypophysectomized recipient rats. The results of these bio-assays were determined by the changes in the weights and cytology of the responsive organs of the recipients. Other similarly extracted pituitaries were studied both cytologically and histochemically. Unextracted pituitaries were both bio-assayed and stained, and these served as reference controls.

MATERIALS AND METHODS

In vitro procedures. In order to establish the validity of some of the methods used, the solubility characteristics, the precipitation and the staining of isolated pituitary hormones[4] were studied by *in vitro* experiments similar to those performed for neurohypophyseal hormones (14) and for insulin (15). It was first established that all 3 glycoprotein hormones were precipitated from an aqueous solution (1 mg./ml.) by the addition of absolute acetone or sublimate-formol. The aqueous solutions were made by the addition of tap water. No attempt was made to quantitate the precipitation or to determine if any biological activity remained in the supernatant. Other precipitants (fixatives) were also tested but they were discarded because they did not irreversibly precipitate the hormones, or they rendered them biologically inactive, or they resulted in poor histological preparations.

The protein precipitates formed by the addition of sublimate-formol to solutions of the hormones or directly to the powdered form of the hormone were collected either on filter paper or in centrifuge tubes. These were found to be insoluble in tap water and in reagents used in dehydrating tissue prior to paraffin embedding, 80%, 95% and absolute ethyl alcohol and chloroform. Deparaffinization was performed on the precipitates as well, and it was found that they remained insoluble in xylene, a graded series of ethyl alcohols (absolute, 95%, 80%, 70%) and water. The precipitates were also insoluble in the reagents used in the PAS method, Lugol's solution, sodium thiosulphate, water, periodic acid and a solution of leucofuchsin. As a result of these procedures, the samples of glycoprotein hormones (FSH, LH and TSH) were stained by the PAS method.

The precipitates formed with absolute acetone, on the other hand, became soluble to

[4] Samples of pituitary hormones were kindly provided by Dr. S. L. Steelman, Armour Laboratories, Chicago, Illinois. FSH-lot K45308R; LH-lot 227–80; TSH-lot 317-52R.

a variable extent when they were subjected to the graded series of alcohols and to water indicating a reversible denaturation. If these precipitates were refixed in sublimate-formol after acetone, they remained insoluble throughout the procedures enumerated.

The solubility of the protein hormones was tested with four solvents. Only FSH was partially soluble in half-saturated ammonium sulfate (1/2 SAS) or in 20.5% sodium sulfate in pH 4.4 acetate buffer. FSH and TSH were not precipitated from solution by the addition of dilute trichloracetic acid (TCA), but LH was precipitated. All the glycoprotein hormones remained in solution upon the addition of dilute aqueous pyridine.

Treatment of donor pituitaries for histology and for bio-assays

The pituitary glands of over 600 donor rats were used in the preliminary and definitive experiments. The present report is based on a study of 226 of these glands obtained from adult male albino rats of the Hisaw strain, 175 to 225 grams in weight. The sample included 147 normal animals, a total of 65 rats which had been castrated 30, 60 and 150 days previously, and 14 rats fed a diet of 0.1% propylthiouracil (PTU) in Purina dog chow for 60 days. The animals were killed by decapitation. The pituitary glands were removed intact and placed in 50 to 100 ml. of fixative or solvent for 24 hours.

Pituitaries fixed in sublimate-formol and absolute acetone for histology and bio-assay, respectively, were considered the controls which contained all the hormones. Glands fixed in these ways were considered comparable since there appeared to be no differences in the amount of PAS-positive material in pituitaries fixed in either sublimate-formol, or in absolute acetone and refixed in sublimate-formol. Absolute acetone could not be used as the histological fixative because some of the glycoprotein hormones were lost during passage through the dilute ethyl alcohols used during the rehydration of histological sections after deparaffinization.

In addition to the control fixatives, 40 solvents were tested of which 4 were utilized in the present study. These are: 1/2 saturated ammonium sulfate (1/2 SAS), 20.5% sodium sulfate in pH 4.4 acetate buffer, 2.5% trichloracetic acid (TCA), and 50% pyridine.

The pituitary glands of a series of 4 to 6 rats were perfused through the proximal aorta with three of the solvents (1/2 SAS, 2.5% TCA and 50% pyridine) and the glands were then removed and fixed for 24 hours in the same solvent. A similar series of glands were placed directly in the solvent without perfusion. No differences were subsequently observed between the two procedures. Consequently, the latter method of extraction by immersion was adopted. The pituitaries, after remaining for 24 hour in the solvents, were refixed for 24 hours in absolute acetone or sublimate-formol for bio-assay and for histological study, respectively. Control pituitaries were placed in absolute acetone or sublimate-formol. The glands for bio-assay were air-dried and stored in a vacuum desiccator containing a drying agent. Two days before the assays were begun, the pituitaries were weighed, ground with a glass homogenizer, suspended in physiological saline, and frozen. The solutions were thawed for use and were kept thereafter at 3° C. For each experiment, the pituitaries were pooled and a final dilution was made so that each ground pituitary was suspended in 4.5 ml. of saline. In the bio-assay of castrate pituitaries, besides the above dilution, a series of half a pituitary per 4.5 ml. of saline was included.

Assay materials and methods

Assays were performed on male and female hypophysectomized Sprague-Dawley rats.[5] From a total of over 300 assay rats used in the preliminary and definitive experiments, the results from 135 rats are included in this paper. These rats were hypophy-

[5] Purchased from Hormone Assay Laboratories, Chicago, Illinois.

sectomized when they were 21 to 23 days of age and the injections of pituitaries to be assayed were begun on the 23rd to 25th days. The recipient rats received 8 subcutaneous injections of 0.5 ml. of saline suspension of pituitaries in the following injection schedule: one injection on the afternoon of the 1st day of the experiment, two injections, morning and afternoon, on the 2nd, 3rd and 4th days, and one injection on the morning of the 5th day. On the afternoon of the 5th day, these as well as uninjected control rats were killed with ether. Other control rats (23 to 25 days of age) were killed two days after hypophysectomy. The first set of control animals killed on the 5th day, i.e., 7 days after hypophysectomy, will be referred to as "conclusion controls," whereas those killed 2 days after hypophysectomy, at the start of the experiment, will be designated as "initial controls" throughout this paper.

The following data were collected from the injected and control assay rats: initial and final body weights; in the males, the weights of adrenals, testes, seminal vesicles and ventral prostates; in the females, those of the adrenals, ovaries and uteri. All of the organs were weighed on a torsion balance sensitive to 0.1 mg. The uteri, prostates and seminal vesicles were expressed of fluid and blotted before they were weighed. The thyroid glands, without weighing them, were removed in a block of tissue taken through the neck region. The only tissues examined histologically from the assay rats were the thyroid glands and the ovaries, both of which were fixed in 10% neutral formalin. These tissues were then dehydrated, embedded in paraffin and sectioned serially at 7 μ. The sections were mounted and stained with either hematoxylin and eosin or chrome alum-hematoxylin and phloxine. Thyroid acinar cell height indices were calculated according to the method of Rawson and Starr (16). The sella turcica of the assay animals was examined with a dissecting microscope for pituitary remnants.

Histological methods for donor pituitaries

The pituitaries of the donors used for histological study were treated as follows: controls were immersed directly in sublimate-formol for 24 hours and the remainder were first placed for 24 hours in one of the solvents and then refixed in sublimate-formol for 24 hours. The glands were dehydrated, embedded in paraffin and 6 consecutive, 3 μ horizontal sections were cut at 2 or 3 different levels. Single sections were mounted on numbered slides which were deparaffinized and immersed in Lugol's solution and thiosulfate to remove the mercuric chloride. Consecutive sections were stained with PAS alone (17), Orange G alone, PAS and Orange G (18), and for sulfhydryl and disulfides (2). Occasional sections were also stained with aldehyde-fuchsin (19) and a trichrome stain (20). By these measures, the same cells could be stained in two consecutive sections by different methods and compared.

RESULTS

The results of the bio-assays of unextracted and extracted pituitaries of normal and experimental animals are summarized in Table 1.

I. *Bio-assays*

Unextracted pituitaries. When unextracted pituitaries of normal, PTU fed, and castrated rats were bio-assayed, the weights of the gonads and accessory sex organs of the recipient assay rats were significantly increased ($P < 0.01$) above the values of the initial controls (compare lines C, F, G with line A, Table 1). The significance of this becomes even greater if reference is made to the conclusion control weights (line B). Note that $\frac{1}{2}$

TABLE 1. BIO-ASSAYS OF UNEXTRACTED AND EXTRACTED RAT PITUITARY GLANDS FROM NORMAL AND EXPERIMENTAL ANIMALS

Line and group	Sex	No. of animals	Mean gain in body wt. (gm.)	Mean ovary wt. ± S.E. (mg.)	Mean uterine wt. ± S.E. (mg.)	Mean testis wt. ± S.E. (mg.)	Mean seminal vesicle wt. ± S.E. (mg.)	Mean prostate wt. ± S.E. (mg.)
A. Initial controls	M	8	—	11.6±1.3	20.3±2.2	263.7±15.2	6.8±0.5	15.7±0.7
	F	4	—					
B. Conclusion controls	M	10	6	10.7±0.9	18.3±0.7	168.8± 8.0	5.9±0.3	5.9±0.5
	F	9	3					
C. Pituitaries of normal adult male rats (acetone-dried), 1 gland/assay animal	M	6	13	71.9±5.6**	97.6±6.4**	423.4±25.4**	20.9±1.7**	23.9±1.2**
	F	6	6					
D. Pituitaries of normal adult male rats extracted with ½ saturated $(NH_4)_2SO_4$ (½ SAS), 1 gland/assay animal	M	5	16	51.1±5.2**	105.2±5.5**	396.1± 8.5**	15.2±1.2**	23.9±0.9**
	F	7	6					
E. Pituitaries of normal adult male rats extracted with 20.5% Na_2SO_4 in acetate buffer at pH 4.4, 1 gland/assay animal	M	5	8	28.0±2.7**	73.0±9.7**	416.1±11.3**	12.6±1.1**	32.4±3.0**
	F	10	9					
F. Pituitaries of adult male rats on 0.1% PTU for 2 months (acetone-dried), 1 gland/assay animal	M	4	5			433.7±11.7**	16.1±1.4**	19.6±1.1**
G. Pituitaries of adult male rats castrate for 2 months (acetone-dried), ½ gland/assay animal	M	7	6	54.4±8.4**	100.7±4.5**	432.9±24.6**	25.2±2.0**	37.5±1.9**
	F	7	6					
H. Pituitaries of normal adult male rats extracted with 2.5% TCA (acetone-dried), 1 gland/assay animal	M	8	7	12.7±1.0	17.5±1.7	251.0±7.1	11.1±0.6**	18.3±1.3
	F	8	8					
I. Pituitaries of adult male rats on 0.1% PTU for 2 months and extracted with 2.5% TCA (acetone-dried), 1 gland/assay animal	M	4	6			245.1±14.0	7.0±0.7	13.6±1.6
J. Pituitaries of adult male rats castrate for 2 months & extracted with 2.5% TCA (acetone-dried), ½ gland/assay animal	M	6	11	18.0±0.6*	20.4±1.3	255.1± 6.9	18.4±1.1**	28.2±2.8**
	F	6	9					
K. Pituitaries of adult male rats castrate for 2 months & extracted with 2.5% TCA (acetone-dried), 1 gland/assay animal	M	3	10			347.9±19.1*	26.6±2.2**	41.2±1.2**
L. Pituitaries of normal adult male rats extracted with 50% Pyridine, 1 gland/assay animal	M	6	6	13.4±0.7	16.5±1.9	192.4±16.8**	7.1±0.3	7.2±0.8**
	F	6	5					

*—P = <0.05 } comparison with initial control values.
**—P = <0.01. }

of a castrate gland (G) produced a response in the gonads of both sexes similar to that of a whole normal gland (C), while the response of the prostate was greatly enhanced. The weight of the prostate and seminal vesicles of animals which received pituitaries from rats fed PTU (F) was slightly less than those that received normal pituitaries (C).

½ SAS and 20.5% sodium sulfate extracted pituitaries. A noticeable reduction in the weight of the recipient ovaries and of the uteri and seminal vesicles occurred as a result of extraction with the sodium sulfate solution (cf. lines E and C, Table 1). However, this extraction was incomplete (compare organ weights in line E with those in line B; if extraction were complete, one would expect values comparable to line B). Half SAS was less efficient in its capacity to extract gonadotropins (compare lines D with E, and D with B). Variations in the ½ SAS treatment of pituitaries by either prior freezing and thawing, or acetone fixation, or alteration of the pH of the ½ SAS solution did not improve the extraction.

2.5% trichloracetic acid (TCA) extracted pituitaries. The results of the bio-assays of normal pituitaries which were extracted with TCA (Table 1, H) showed an extraordinary decrease in the recipients' ovarian, uterine and testicular weights when compared to the results obtained with the unextracted normal glands (C). These values were similar to those of the initial or conclusion controls. The TCA extracted normal pituitaries (H) contained enough gonadotropin to maintain the testis weight and that of the prostate above that of the conclusion control (B) values. The weight of the seminal vesicles (H) was significantly higher than the initial controls, but it still had not attained the level of response which was demonstrated after the administration of an unextracted, normal pituitary (C).

The results of the bio-assays of pituitaries from animals fed 0.1% PTU (both unextracted, (F) and TCA extracted (I)) gave similar responses for the testis weight as the normal control unextracted (C) and extracted pituitaries (H), respectively. The response of the seminal vesicles and the prostate to the extracted pituitaries (I) was diminished from the values obtained with normal extracted pituitaries (H) or PTU unextracted glands (F).

The bio-assays of the extracted pituitaries of castrate animals (J) showed a decrease in the ovarian, uterine and testicular weights in comparison to the unextracted castrate pituitaries (G) so that the uterine and testicular weights were the same as the initial controls (A) and the ovarian weights were somewhat larger. On the other hand, the seminal vesicle and prostate weights were elevated to a statistically significant value with reference to the initial controls, but were still below those obtained with the unextracted castrate pituitary. When the results of the TCA extracted normal glands (H) and the TCA extracted castrate glands (J) are compared, the amount of gonadotropin present in a half of a castrate gland

gave a greater response in the seminal vesicles and prostate weights than a whole extracted normal gland, whereas the uterine and testicular weights in this series gave about the same response. In this series, the response of the ovaries was somewhat greater. When a whole extracted castrate pituitary (K) was assayed, the effects on the accessory organs of the male were markedly greater and, in addition, the testis weight increased significantly.

50% pyridine extracted pituitaries. Normal pituitary glands extracted with 50% pyridine (L) contained virtually no gonadotropin, since the bio-assay results were almost identical with the conclusion controls (B) and less than the initial controls (A). In a small series not included in Table 1, pituitaries of castrated animals extracted with 50% pyridine contained a little gonadotropin as indicated by a slight increase in the seminal vesicle and prostate weights over that of the initial controls.

Thyroid cell height indices. Inspection of Table 2 reveals that injection of an unextracted normal pituitary caused an appreciable increase in the cell height index over the initial control value. The unextracted pituitaries from PTU fed animals did not raise the cell height indices above the level of the conclusion controls. Similarly, there was no increase in the cell height indices in the groups which received pituitaries extracted with 2.5% TCA or 50% pyridine from either normal or PTU fed animals. Although not included in Table 2, the cell height indices of animals receiving pituitaries extracted with $\frac{1}{2}$ SAS or 20.5% sodium sulfate were similar to those of unextracted normal pituitaries. (It should be noted that the cell height indices of the thyroids of both untreated and treated hypophysectomized rats were lower than those reported in the literature, but this was a constant finding in these rats and the variation was small.)

Ovarian histology. The histological appearance of the ovaries of the assay animals is shown in Figures 1 to 7. The ovaries of the initial control rats (Fig. 1) contained many small, densely packed follicles and little interstitial tissue. Seven days after hypophysectomy (conclusion controls)

TABLE 2. THE THYROTROPIC ACTIVITY OF PITUITARY GLANDS FROM NORMAL AND EXPERIMENTAL ANIMALS PRIOR TO AND AFTER EXTRACTION WITH 2.5% TRICHLORACETIC ACID AND 50% PYRIDINE

Group	No. of animals	Mean thyroid acinar cell height in $\mu \pm$S.E.
Initial controls	5	3.25 ±0.07
Conclusion controls	5	2.53 ±0.21
Normal male pituitary (acetone-dried)	4	3.79 ±0.30
PTU fed male pituitary (acetone-dried)	4	2.66 ±0.08
Normal male pituitary extracted with 2.5% TCA (acetone-dried)	8	2.67 ±0.15
PTU fed male pituitary extracted with 2.5% TCA (acetone-dried)	4	2.49 ±0.29
Normal male pituitary extracted with 50% pyridine (acetone-dried)	6	2.48 ±0.27

(Fig. 2), there was a decrease in the number of follicles, although some of these were larger and contained a thicker granulosa. There was no real increase in the amount of interstitial tissue, although there seemed to be a slight increase in the stroma. After treatment with a normal unextracted pituitary, the ovaries contained many large Graafian follicles, with hyperplastic thecae and a greater amount of intersititial tissue (Fig. 3). Administration of a normal pituitary extracted with TCA caused a remarkable increase in the amount of interstitial tissue and the luteinization of some of the follicles. The remainder of the follicles in these ovaries were similar to those of the conclusion controls and, therefore, unstimulated (Fig. 4). Injection of a normal pituitary extracted with 50% pyridine did not elicit a response (Fig. 7), and the ovaries resembled those of the conclusion controls (Fig. 2). The effects of administration of half of an unextracted castrate pituitary (Fig. 5) or of a TCA extracted castrate gland (Fig. 6) produced the same histological effects as the unextracted (Fig. 3) and extracted (Fig. 4) normal whole pituitary, but the glands of castrated animals contained more gonadotropin. The increase in interstitial tissue resulting from the injection of one half of a TCA extracted castrate pituitary was indeed profound (Fig. 6).

Although there was a suggestive decrease in the ovarian weights of the assay animals which received pituitaries extracted with $\frac{1}{2}$ SAS as compared with animals receiving unextracted normal glands (*cf.* lines D with C, Table 1), the histological appearance of these ovaries was similar to that of ovaries of animals which received the unextracted pituitaries. The ovaries of animals receiving pituitaries extracted with sodium sulfate showed follicular growth which was less than, and intersititial growth which was equal to, that produced by unextracted pituitaries. Thus, the ovaries appeared histologically as intermediate between Figures 2 and 3.

II. *Cytology of the Anterior Pituitary Gland*

In the *normal* rat's anterior pituitary, many PAS-positive cells are found throughout the gland, whereas some are regionally concentrated.

The PAS-positive cells can be divided into 3 types on the basis of their staining reactions, shapes, locations and responses to endocrine imbalances. Two of these cell types were classified as "gonadotrophs" by Purves and Griesbach (21), as "delta" cells by Halmi (22), as belonging to some of the "mucoid" cells by Pearse (1), and as equivalent with some of the classical "basophiles" by many other workers. Of the "gonadotrophs," one type which is coarsely granulated is concentrated mainly in an area called the "sex zone" (adjacent to the pars intermedia), whereas a few of this type are more peripherally scattered. This cell type is said to be concerned with the production of FSH (23). The other type of "gonadotroph," which is finely granulated and centrally located, is thought to produce LH (24).

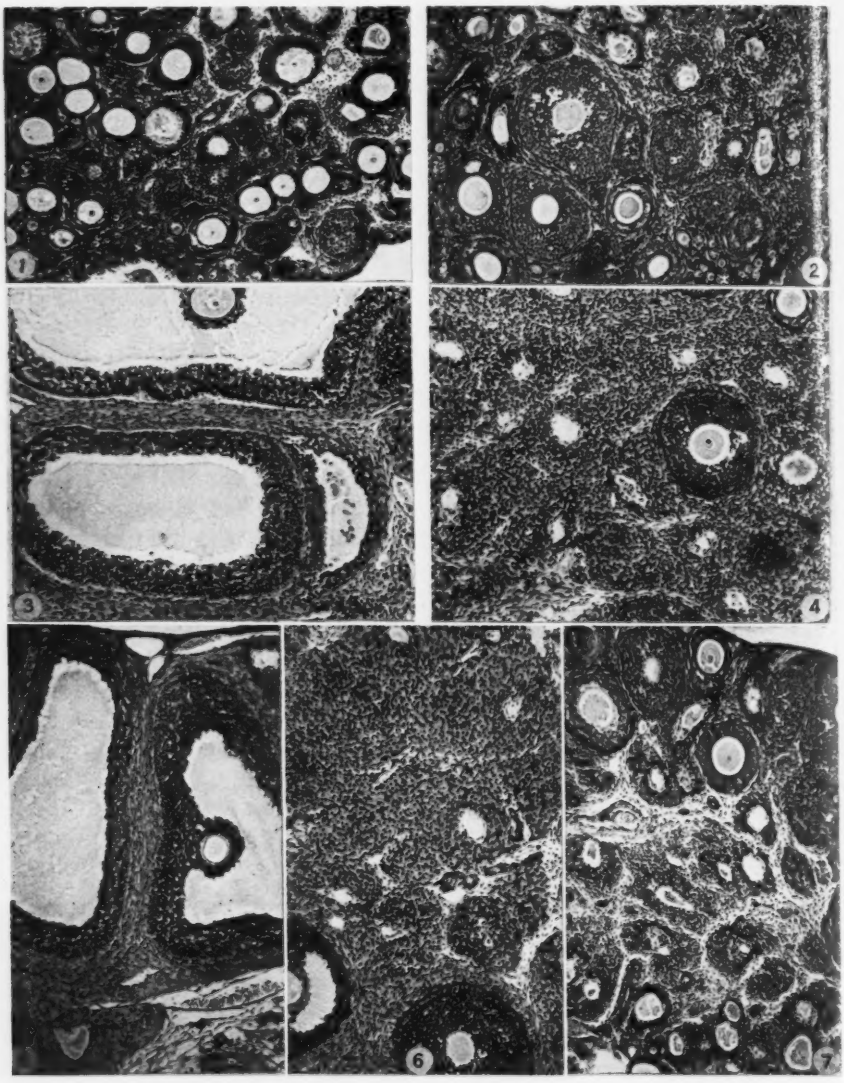

PLATE 1

These figures are representative photomicrographs of histological sections of ovaries of hypophysectomized assay animals. H and E. All ×40.

FIG. 1. Untreated initial control. Numerous small follicles and little interstitial tissue.

FIG. 2. Untreated conclusion control. Reduction in the number of small follicles and little interstitial tissue.

FIG. 3. Injected with unextracted normal pituitary. Many large, Graafian follicles, hyperplastic thecae and increased interstitial tissue. Evidence of stimulation by FSH and LH.

A third type of PAS-positive cells ("thyrotrophs" of Purves and Griesbach (25), the "beta" cells of Halmi (26), some of the "mucoid" cells of Pearse (1) and some of the classical "basophiles" of other workers), which also stains selectively with aldehyde-fuchsin under certain conditions, is considered to produce TSH (26).

Figure 8 illustrates the PAS-positive cells of the lateral lobe of a normal rat's anterior pituitary. The PAS-positive cells in the "sex zone" (Fig. 9) and at the periphery of the anterior lobe were usually round or oval and either heavily or moderately granulated and therefore intensely or lightly stained. The granules in these cells appeared either coarse (P) or fine (L) (Fig. 11). The PAS-positive cells in the "central zone" (Figs. 10, 12) tended to be more angular in shape, contained, for the most part, fine granules and were less intensely PAS-positive (L). In addition, large, heavily granulated, intensely PAS-positive polygonal cells (T) were scattered throughout the lateral lobe, including the "sex zone," and in the "central zone" where they were most abundant. These PAS-positive cells, because of their shape and the fact that they stained selectively with aldehyde-fuchsin, are regarded as "thyrotrophs." We would like to stress that the separation of PAS-positive cells on the basis of intensity of staining and type of granulation is not absolute but relative.

In *normal* pituitaries *extracted with 2.5% TCA*, there was an extreme reduction in the amount of PAS-positive material and a slight decrease in the number of PAS-positive cells (Figs. 13, 14, 15, 16, 17). The cells with residual PAS-positive material were located both in the peripheral (Figs. 14, 16) and central (Figs. 15, 17) regions of the gland. There were cells which appeared to us to be "basophiles" on the basis of their size, shape and location which were devoid of PAS-positive material. No aldehyde-fuchsin positive cells were seen in these glands.

In the *unextracted* pituitaries of *castrate* animals, the well-known castrate picture developed (i.e., increased amount of PAS-positive material as well as an increased number of these cells with an enlarged Golgi apparatus and colloid-filled vacuoles (Figs. 18, 20, 21). In all regions of the gland, there was an intermingling of cells in which the PAS-positive

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FIG. 4. Injected with 2.5% trichloracetic acid extracted normal pituitary. Few follicles and greatly increased amount of interstitial tissue. Evidence of stimulation by LH only.

FIG. 5. Injected with unextracted castrate pituitary (1/2 gland). Similar to Fig. 3 in intensity of stimulation by FSH and LH.

FIG. 6. Injected with 2.5% trichloracetic acid extracted castrate pituitary (1/2 gland). Few follicles and very greatly increased amount of interstitial tissue. Evidence of stimulation by LH only.

FIG. 7. Injected with 50% pyridine extracted normal pituitary. Few small follicles and little interstitial tissue. Very similar to Fig. 2. No evidence of stimulation by FSH and LH.

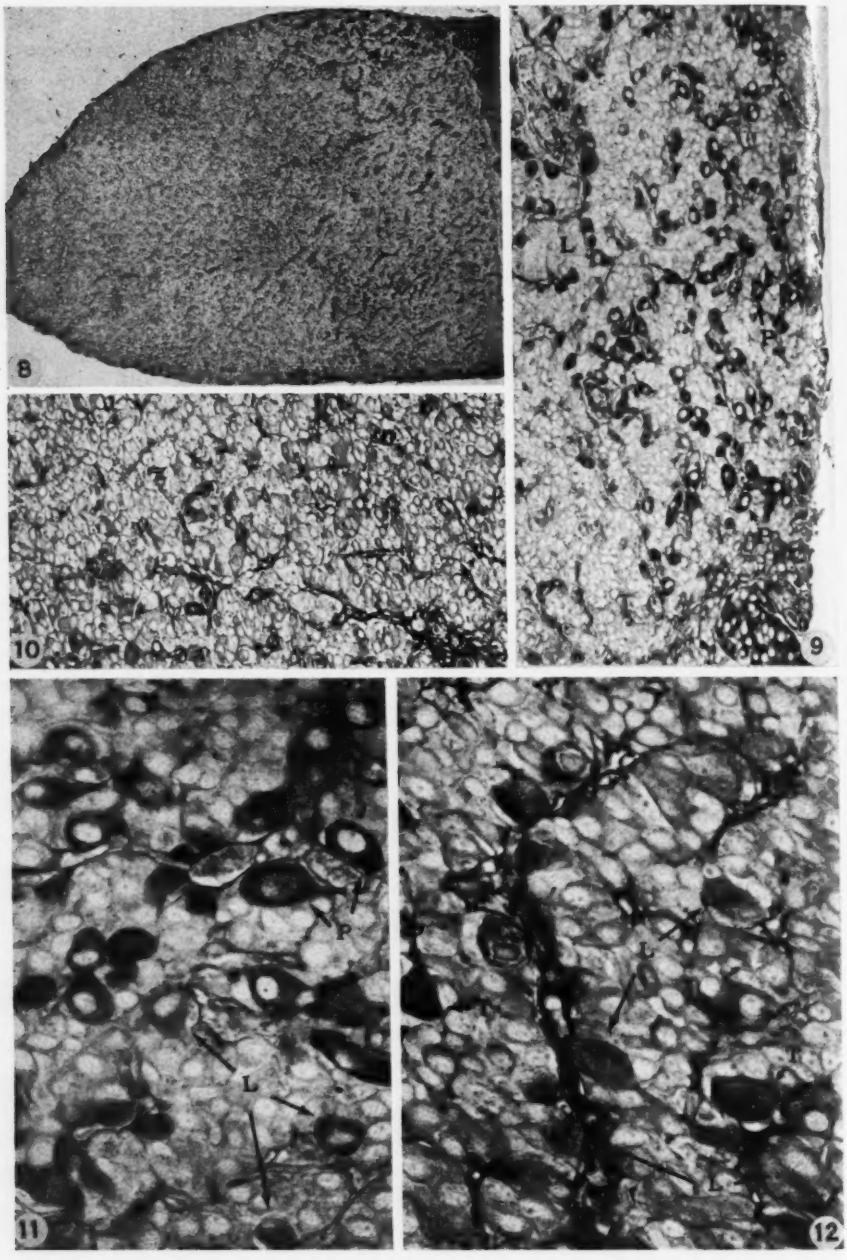

PLATE 2

All figures on this plate are representative photomicrographs of histological sections of the anterior pituitary glands of normal male rats, stained by the PAS method.

material varied in amount, intensity of staining, distribution within the cell and in the size of its granules (Figs. 20, 21, 24, 26).

In the *TCA extracted* pituitaries of *castrate* animals, a great reduction in the amount of PAS-positive material was seen (Figs. 19, 22, 23, 25, 27). However, it must be stressed that the PAS-positive material which remained after extraction was present in cells which were diffusely scattered throughout the gland (Figs. 22, 23, 25, 27). It was evident that some obvious "basophiles" contained no PAS-positive material (Figs. 25, 27) and that in most of the remaining PAS-positive cells, the amount of stainable granular material was diminished when compared to the unextracted castrate glands (cf. Figs. 20, 21 with 22, 23, and, 24, 26 with 25, 27).

Examination of *unextracted* pituitaries from *PTU fed* animals revealed that there were no aldehyde-fuchsin positive granules in any of the cells. Some cells were greatly enlarged and contained little PAS-positive granular material. Smaller cells which contained moderate amounts of PAS-positive material were observed in the "sex zone." "Acidophiles" were devoid of granules and were unstained. In the *TCA extracted* pituitaries of the *PTU fed* animals there was frequently less PAS-positive material than in the normal TCA extracted glands.

In the pituitaries *extracted* with *50% pyridine*, no cells stained with PAS and aldehyde-fuchsin (Figs. 28, 29). These extracted glands contained cells which were swollen and almost completely lacking in cytoplasm (Fig. 29). The distribution of the cells conformed to the localization of the PAS-positive cells in the glands (Fig. 28), but the number of extracted cells was greater than the number of PAS-positive cells. After this extraction, the "acidophiles" were somewhat decreased in number and stained moderately with acid dyes or for sulfhydryl and disulfide groups.

FIG. 8. A lateral half of the anterior pituitary. Note the distribution of the PAS positive cells in this section, particularly in the "sex zone" at the upper right, adjoining a portion of the pars intermedia. ×50.

FIG. 9. A higher magnification of the "sex zone" which reveals two types of PAS-positive cells. These are: the more coarsely granulated, darker-staining cells located at the periphery (at the right, —P) and the finer granulated, lighter-staining cells which are scattered from the periphery towards the central region (at the left,—L). ×230.

FIG. 10. A higher magnification of the "central zone" which shows the many lighter-staining, more finely granulated cells and an occasional heavily-staining, polygonal cell. ×230.

FIG. 11. The cytological appearance of the coarsely granulated cells (P) at the periphery can be compared with that of some of the lighter-staining cells (L), both of which are clearly seen in the "sex zone." ×670.

FIG. 12. The PAS-positive cells of the "central zone" also exhibit two types. These are: the finer-granulated, lighter-staining cells (L) and the heavily-staining, polygonally-shaped cells (T), which are regarded as "thyrotrophs" or "beta" cells. ×670.

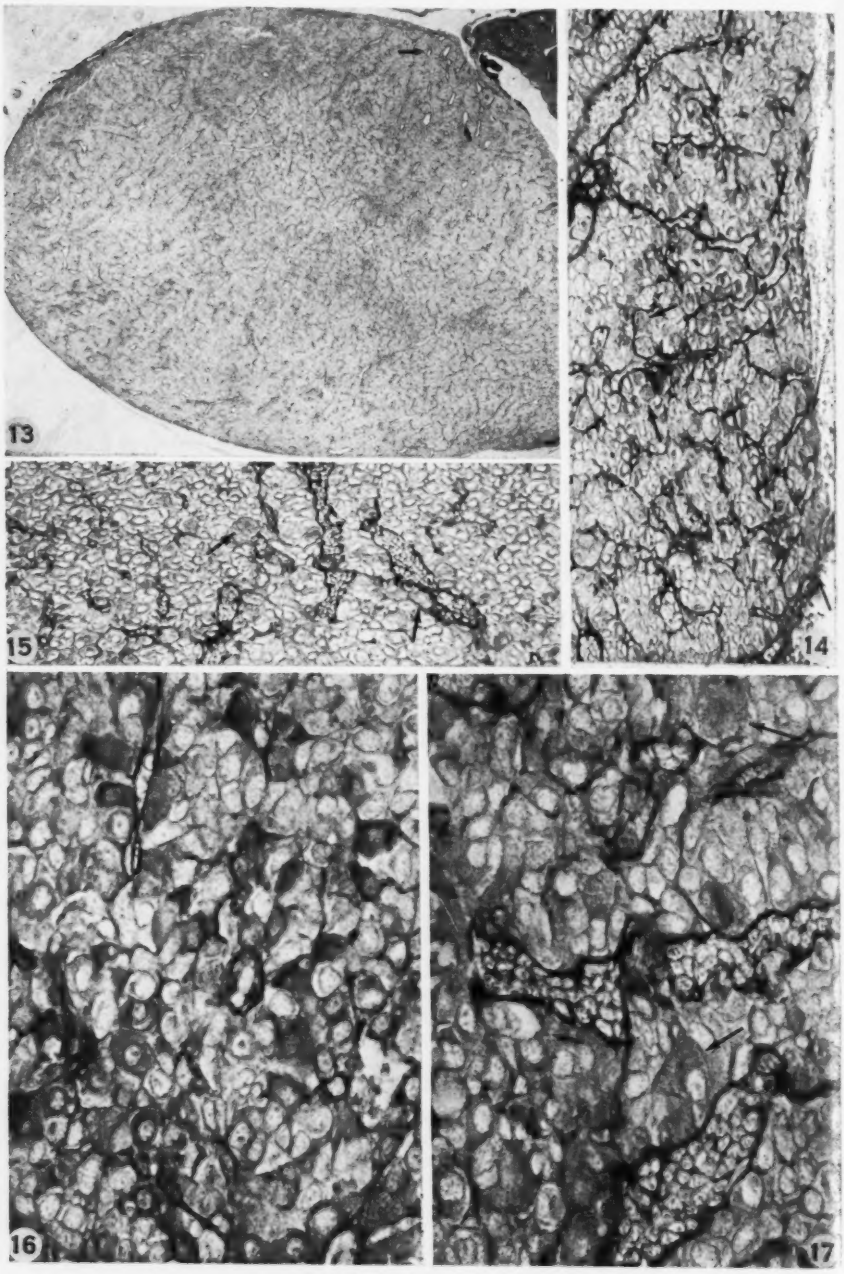

PLATE 3

The cytology of the pituitaries *extracted* with $\frac{1}{2}$ *SAS* and *20.5% sodium sulfate* was extremely difficult to interpret. Following the latter procedure, cellular shrinkage was so pronounced that differences in the amount of stainable material in the PAS-positive cells could not be ascertained. However, in two out of 14 glands so treated, there was an impression of a decrease in PAS-positive material. If shrinkage artifacts were overlooked, the intensity of the staining of some of the peripheral and central PAS-positive cells appeared about the same.

DISCUSSION

The present experiments demonstrate the sites of glycoprotein hormones in the rat's anterior pituitary gland by the combined use of several techniques. We have performed *in vitro* experiments on the solubilities, precipitation and staining of samples of purified glycoprotein hormones. In addition, pituitary glands have been subjected to selected solvents, designed to remove one or more of the glycoprotein hormones, after which they were studied by histological and bio-assay methods. Protein solubilities as a histochemical tool have been little used previously (27, 28).

Our results indicate that the PAS-positive material localized in the "basophiles" of the anterior pituitary represents the glycoprotein hormones FSH, LH and TSH. This general conclusion had been suggested by others (1, 23, 24, 25, 26, 29) on histological criteria.

We have used as our bio-assay and histochemical controls pituitaries fixed in absolute acetone and sublimate-formol, respectively, because all of the hormones appear to be retained following the use of these two fixa-

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All figures on this plate are representative photomicrographs that show histological sections of the anterior pituitary glands of normal male rats, stained by the PAS method. These glands were extracted with 2.5% trichloracetic acid (TCA) prior to histological fixation.

FIG. 13. The appearance of the lateral half of the anterior pituitary after TCA extraction (compare with Fig. 8). PAS staining is considerably reduced. Many weakly-staining cells can be seen throughout the gland, particularly in the area of the "sex zone," which show the portal vessels to advantage (arrows, upper right). ×50.

FIG. 14. The weakly-staining, PAS-positive cells in the "sex zone" can be seen at the periphery (at the right, arrows) and also some that are oriented towards the central region (at the left, arrows). ×230.

FIG. 15. Only a few weakly-staining, PAS-positive cells are visible at this magnification in the "central zone" (arrows). ×230.

FIG. 16. A higher magnification of the weakly-staining, PAS-positive cells of the "sex zone." ×670.

FIG. 17. A higher magnification of the weakly-staining PAS-positive cells of the "central zone." In some cells (lower arrows) the suggestion of a coarse as well as a fine granular content in the cytoplasm can be made out, whereas in others (top arrow) only a uniform weak granulation can be discerned. ×670.

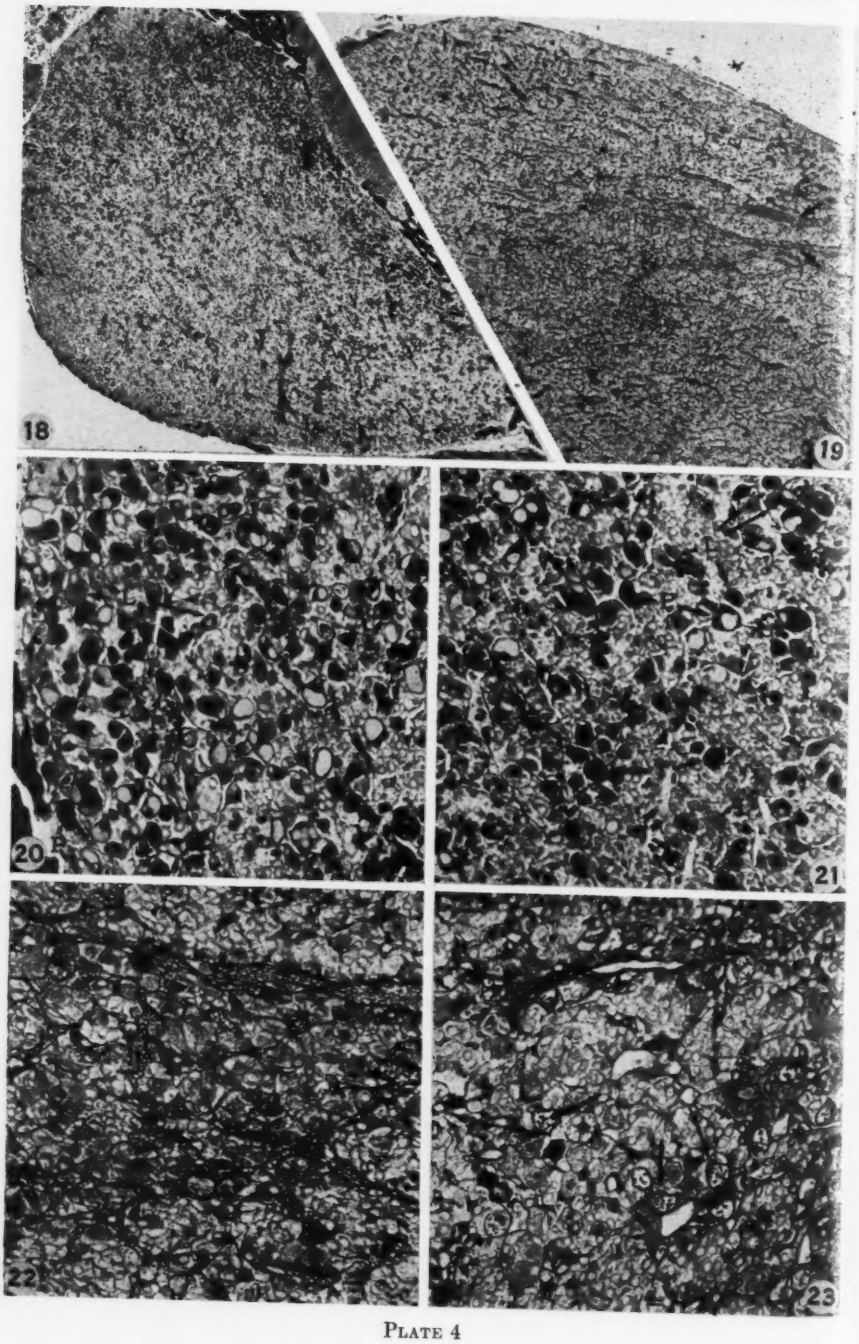

PLATE 4

tives. FSH and TSH were removed from pituitaries of normal and castrated rats by extraction with 2.5% TCA, as indicated by the changed histology of the recipient ovaries and thyroids, and the weights of the ovaries, uteri and testes of the assay animals. Normal pituitaries extracted with 2.5% TCA contained no aldehyde-fuchsin-positive cells. This finding lends support to the observation (25, 26) that the aldehyde-fuchsin-positive cells contain TSH. The absence of these cells in the unextracted pituitaries of PTU fed rats also confirms this observation.

The PAS-positive material which remains in the gland after extraction with 2.5% TCA is LH, as demonstrated by the weight changes of the ventral prostate and the altered ovarian histology of the assay rats. The LH-containing cells, revealed by the PAS-positive material remaining after 2.5% TCA extraction, were not regionally concentrated in the "central zone" as maintained by Purves and Griesbach (24), but were scattered throughout the gland in a pattern conforming to the distribution of PAS-positive cells in the normal pituitary.

If one holds that FSH is predominantly localized in the "basophiles" of the "sex zone" (23) and accepts that after TCA extraction the number of PAS-positive cells is relatively the same as in unextracted glands although

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Figures 18 to 27 are representative photomicrographs that show histological sections of the anterior pituitary glands of male rats which had been castrated 60 days previously, stained by the PAS method. Some of the pituitaries were extracted with 2.5% TCA, prior to histological fixation.

FIG. 18. Note the increased number and size of the PAS-positive cells and also their distribution after castration in this slightly obliquely cut lateral half of a pituitary gland ("sex zone" to upper right). Compare with normal gland (Fig. 8). ×42.

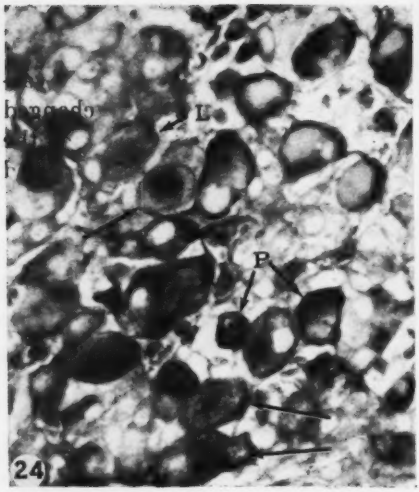
FIG. 19. A lateral half of a pituitary extracted with TCA, which shows the considerable reduction in number and in staining intensity of PAS-positive cells. As in the normal gland extracted with TCA, there are many weakly staining PAS-positive cells scattered throughout the gland, particularly in the "sex zone" and along the portal vessels (arrows, upper right). ×42.

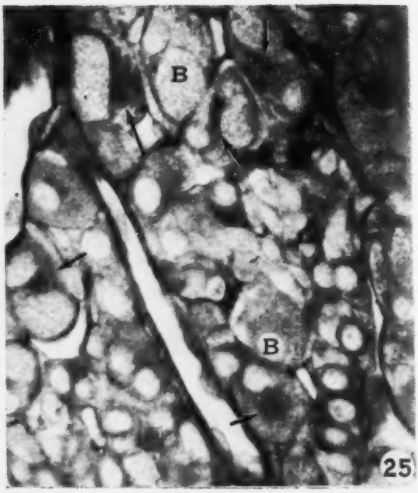
FIG. 20. Two types of PAS-positive cells can be distinguished in the "sex zone" of the castrate; the heavily-staining, coarsely granular, peripheral cells (P) and the lighter-staining, more finely-granular cells (L). Many of them contain the "signet ring" vacuole filled with colloid. ×230.

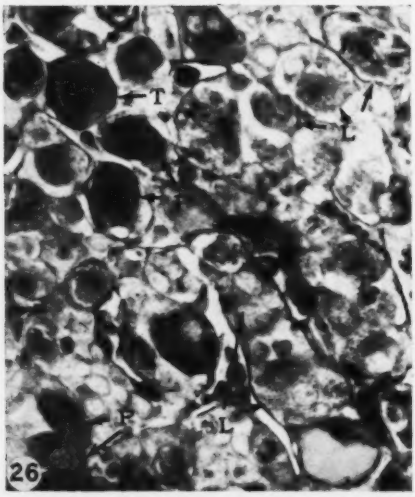
FIG. 21. Three types of PAS-positive cells can be discerned in the "central zone" of the castrate; the larger, polygonal, more heavily-staining cells (T), the lighter-staining, more rounded cells (L) and the large, darker-staining, somewhat angular or ovoid cells (P), some of which contain the "signet ring" vacuole. ×230.

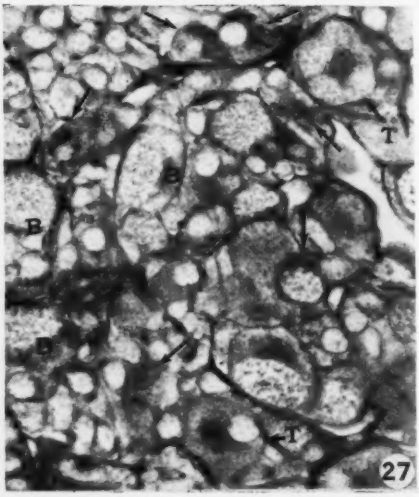
FIG. 22. The "sex zone" of a castrate which was extracted with TCA reveals many weakly-staining, PAS-positive cells. Coarsely-granulated, heavily-staining cells are absent. ×230.

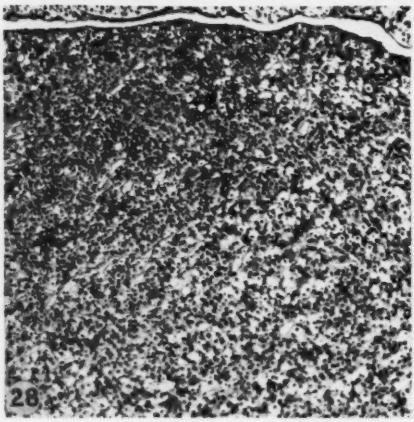
FIG. 23. The "central zone" of a castrate which was extracted with TCA similarly shows many weakly-staining, PAS-positive cells. Many vacuolated cells, basophiles, (arrows) contain residual precipitate of particulate protein in them, a result of the TCA extraction. ×230.











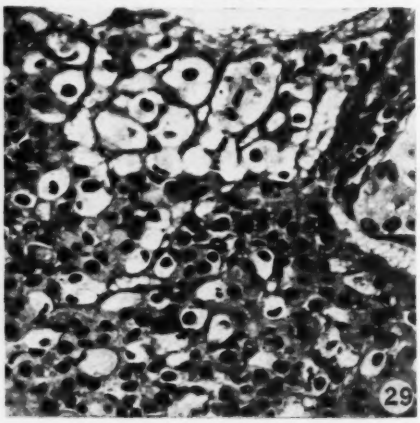


PLATE 5

the intensity of their staining has diminished (cf. Figs. 9 with 14, and 11 with 16), then one would have to conclude that FSH and LH were present in the same cells. Similarly, if one also holds that LH is localized in the "central zone" (24), then the slight decrease in staining of the cells of the "central zone" after TCA extraction could be due to the loss of FSH and/or TSH. Since only a very slight loss of LH occurs after TCA extraction, our findings might also be interpreted as indicating that only LH is contained in the cells of the "central zone." The occasional "basophile" which does not contain PAS-positive material after extraction with TCA could be the cell of origin of either FSH or TSH or both hormones. In our opinion, it is unlikely that FSH and TSH are produced in the same cells in view of the abundant morphological studies that have clearly separated these hormones into distinct PAS-positive cell types (21, 22, 23, 25, 26).

The evidence that FSH and LH occur in the same cells is good. This is readily apparent when the amount of PAS-positive material in the cells of the "sex zone" of unextracted and TCA extracted normal pituitaries is compared. This finding is further strengthened when all regions of

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FIG. 24. A higher magnification of the PAS-positive cells of the "sex zone" of the castrate illustrates the cytology of the heavily-staining (P) and lighter-staining (L) cells. Note that many PAS-positive cells contain both types of granules (arrows). ×670.

FIG. 25. After extraction with TCA, the "sex zone" of the castrate appears almost unstained. Some large cells, "basophiles" (B), appear devoid of stainable material whereas in other "basophiles," conspicuous, intensely-staining, large granules can be seen in the cytoplasm (arrows). ×670.

FIG. 26. A higher magnification of PAS-positive cells of the "central zone" of the castrate shows, to the upper left, a large, polygonal cell which may be regarded as a "thyrotroph" (T) or "beta" cell; some elongate, occasionally angular, heavily-staining cells (P); and many lighter-staining cells (L), some of which contain rather large, intensely-staining granules (arrows). ×670.

FIG. 27. After extraction with TCA, the "central zone" of the castrate contains many large cells; "basophiles" (B), the cytoplasm of which is devoid of PAS-positive granules; two large, polygonal cells (T) which may be regarded as "thyrotrophs"—the cytoplasm of the lower cell contains some large, intensely-staining granules, whereas in the upper cell, these granules are absent; and many rounded or angular cells which contain faintly-staining cytoplasm and large, intensely-staining granules (white-bordered arrows). ×670.

Figures 28 and 29 are photomicrographs of histological sections of the anterior pituitary glands of normal male rats, stained by the PAS method. These glands were extracted with 50% pyridine prior to histological fixation.

FIG. 28. A lateral portion of the anterior pituitary (pars intermedia above). There appears to be a greater number of the "extracted" cells after treatment with 50% pyridine than can be accounted for by the number of PAS-positive cells alone. ×83.

FIG. 29. The "sex zone" showing the large number of "vacuolated" or "extracted" cells. The slight residual cytoplasm that remains in these cells after the extraction procedure is not PAS-positive. (Pars intermedia at the right.) ×335.

unextracted and TCA extracted pituitaries of castrated rats are contrasted. In the pituitary of the castrated animal, the increase in gonadotropin following castration is correlated with an increase in PAS-positive material. In TCA extracted glands of castrated rats, the decrease in the amount of PAS-positive material in virtually all of the "basophiles" may be correlated with the loss of FSH activity.

The findings in both the bio-assays and in the histological material clearly indicate that the procedure of extracting the pituitaries with 2.5% TCA, refixing in sublimate-formol and then staining by the PAS method provides a histochemical method for the demonstration of LH.

After extraction of the pituitaries with 50% pyridine, glycoprotein disappears histochemically from the "basophiles" and FSH, LH, and TSH activities are not demonstrable upon bio-assay. However, the number of extracted cells emptied of cytoplasmic constituents is greater than the number of PAS-positive cells. Two explanations may possibly account for this disparity. First, the cells in the normal unextracted pituitary which are poorly granulated and considered to be "chromophobes" or potential "basophiles" may have been extracted, or secondly, some "acidophiles" may have been completely extracted with the loss of some of the simple protein hormones while the glycoproteins were being extracted from the "basophiles."

The bio-assays indicate that 20.5% sodium sulfate, and to a lesser extent $\frac{1}{2}$ SAS, removes some FSH but no LH or TSH. Histological correlations are unreliable with these two solvents because of the shrinkage artifacts produced in the pituitary cells.

The methods and rationale described in this paper, by which the glycoprotein hormones have been localized in the PAS-positive cells of the anterior pituitary, are being applied in further studies that have as their aim the identification and the cellular localization of the simple protein hormones of the hypophysis (30) and of the glycoprotein hormones of the placenta.

SUMMARY

This investigation demonstrated the localization of glycoprotein hormones (FSH, LH and TSH) in the cells of the anterior pituitary gland of rats by combined use of differential protein solubilities, staining procedures and bio-assays.

The effects of four solvents upon the structure of the pituitary cells and upon their glycoprotein hormone contents were studied in normal, castrate and propylthiouracil fed rats. These results were compared with similar findings of unextracted control pituitary glands.

The four solvents contributed to a differential analysis of the localization of the glycoprotein hormones of the anterior pituitary gland.

a. Both half-saturated ammonium sulfate and 20.5% sodium sulfate

in pH 4.4 acetate buffer extracted some FSH as determined by bio-assays, leaving LH and TSH. Histological correlations with these solvents were not apparent because of shrinkage artifacts.

b. 2.5% trichloracetic acid extracted FSH and TSH completely, but did not remove LH as determined by bio-assays. Histologically, there was a reduction of staining in those cells which contained PAS-positive material ("basophiles") and an absence of aldehyde-fuchsin staining. These histological changes are obviously due to the removal of FSH and TSH, whereas the residual PAS reaction is attributable to LH. Consequently, this whole procedure provides a histochemical method for the identification of LH.

c. 50% pyridine extracted all three glycoprotein hormones as determined by bio-assays. Histologically, all PAS-positive material was extracted from the gland and the "basophiles" appeared vacuolated.

The results prove that all three glycoprotein hormones are localized in PAS-positive cells ("basophiles"). TSH is contained in glands that possess aldehyde-fuschsin-positive cells. LH is found in PAS-positive cells scattered throughout the gland and it is not regionally confined to the "basophiles" of the "central zone." LH is frequently associated with FSH, especially in PAS-positive cells of the "sex zone" of normal rats, whereas this association is less apparent in cells of the "central zone."

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INFLUENCE OF ADRENAL CORTEX ON GASTRIC PEPSINOGEN IN THE RAT[1]

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HYPOPHYSECTOMY, adrenalectomy, and the administration of cortisone have been shown to cause changes in the pepsin content of the gastric juice of rats, which paralleled changes in the size and zymogen content of the chief cells of the gastric mucosa (1, 2). Since histochemical techniques generally allow of only rough quantitative estimates of the enzyme content of tissues, we have made direct measurements of the pepsinogen content of the whole stomach in rats, in a study of the effects of adrenocortical steroids on gastric pepsinogen. The results obtained have shown that while various adrenocortical steroids will restore to normal the gastric pepsinogen depressed by adrenalectomy, none appeared capable of raising this content above normal. ACTH, however, did cause an increase in pepsinogen above normal.

METHODS

Adult male albino rats of the Sprague-Dawley strain were used, and were fed on Purina Chow. In addition, adrenalectomized rats were given 0.9% saline to drink except where sodium depletion was attempted.

Adrenalectomy was performed by the dorsal route, under ether anesthesia. Sham adrenalectomy consisted of visualization of the adrenals without their removal. These sham-adrenalectomized animals comprised the control and the ACTH-treated groups and others labelled "intact" in the tables.

Hypophysectomized animals were purchased and adrenalectomized one week after the initial operation, and one week prior to being sacrificed. All the other experiments ran for 7 days preceding sacrifice, except for the sodium and potassium depletion experiments, which ran for 2 weeks.

The hormones were prepared for injection as follows: Corticosterone was dissolved in absolute alcohol, 10 mg. per ml., and further diluted with water to give the appropriate dose in 1 ml.; commercial suspensions of cortisone acetate and hydrocortisone acetate (25 mg. per ml.) were further diluted with water as necessary; corticotropin gel (ACTH) and desoxycorticosterone acetate (DCA) in oil were used as supplied commercially.

Injections of steroids and ACTH were given intramuscularly into the thighs, while injected supplements of sodium and potassium chloride were administered subcutaneous-

Received April 7, 1956.

[1] This study was supported in part by a grant from the research funds of the Horace H. Rackham School of Graduate Studies of the University of Michigan.

[2] Member of Howard Hughes Medical Institute.

ly over the back. All injections were started at the time of adrenalectomy and given for 7 days in each group; half the daily dose was administered at intervals of 12 hours. The last injections were given 4 to 6 hours before the animals were sacrificed with ether. The stomachs were excised, washed in water to remove any food particles, and examined for gastric erosions. Whole stomachs were homogenized individually in 100 ml. of distilled water in a Waring blendor for five minutes and the pepsinogen content of aliquots, diluted ten times in 0.001N HCl, was determined by the hemoglobin method previously described for urinary pepsinogen (4). Results are expressed as units of pepsin (P.U. per stomach.

RESULTS

Effects of Adrenalectomy and Steroid Administration (Table 1). Adrenalectomy significantly reduced the pepsinogen content of the gastric mucosa

TABLE 1. EFFECTS OF ADRENALECTOMY ON RAT STOMACH PEPSINOGEN CONTENT WITH AND WITHOUT ADRENOCORTICAL STEROID REPLACEMENT. ALL EXCEPT THE CONTROL RATS WERE ADRENALECTOMIZED

Type of experiment	No. of rats	Mean weight		Mean pepsinogen (P.U./stomach)	S.D. ±	Significant difference* Compared to	
		Initial, gm.	Loss in 7 days, gm.			Control P	Adrenalectomy P
Control: Sham-Adrenalectomy	45	338	16	17,720	8,667	—	<.001
Adrenalectomy only: With or without saline drinking water—full diet	45	319	22	10,280	6,505	<.001	—
Adrenocortical extract:							
1 ml./day	8	383	34	11,500	4,620	>.05	>.5
2 ml./day	11	308	35	15,490	8,450	>.5	<.05
Corticosterone:							
0.25 mg./day	8	316	60	9,812	2,748	<.02	>.5
2.5 mg./day	21	327	30	17,895	9,551	>.5	<.01
D.C.A.:							
2.5 mg./day	6	375	(+2)	7,250	5,098	<.02	>.3
5.0 mg./day	15	370	41	14,473	5,300	>.5	<.05
Cortisone:							
0.25 mg./day	6	328	40	8,110	1,889	<.02	>.4
2.5 mg./day	14	339	53	15,714	7,300	>.5	<.05
25 mg./day	12	325	83	4,625	2,182	<.001	<.01
Hydrocortisone:							
2.5 mg./day	14	350	48	17,628	4,995	>.5	<.01

* Significance of difference of means (Students "t" test).

of the rat, while adequate doses of adrenal cortex extract (ACE, 2 ml./day) restored the pepsinogen approximately to normal, as did the following doses of individual steroids: corticosterone acetate (cpd. B), 2.5 mg./day; cortisone acetate (cpd. E), 2.5 mg./day; and desoxycorticosterone acetate (DCA) in oil, 5 mg./day. The same steroids, in doses which were comparable with the amounts present in the 2-ml. dose of adrenal cortex extract used, failed to restore the pepsinogen to normal. Neither adrenal cortex extract nor any of the doses of the individual steroids used was found to increase the gastric pepsinogen content above normal.

Effects of ACTH (Table 2). In contrast with the effects of the individual

steroids in the adrenalectomized rats, ACTH, in intact rats resulted in an increase in gastric pepsinogen to a level significantly above normal. That this effect was mediated by the adrenals was shown by the observation that in adrenalectomized rats ACTH lost its stimulant properties, and in fact profoundly reduced gastric pepsinogen.

The Depressant Effects of Massive Doses of Cortisone (Table 3). Very large doses of cortisone (25 mg./day) given to adrenalectomized rats depressed pepsinogen levels significantly below those found in adrenalectomized rats not so treated. These animals were profoundly anorexic and obviously malnourished, and probably suffered from potassium deficiency caused by an increased excretion of potassium. In addition, it is possible that there was sodium deficiency due to diminished intake of sodium.

To determine whether the reduced food intake may have been responsible for the reduction of gastric pepsinogen, both intact and adrenalec-

TABLE 2. THE EFFECTS ON RAT GASTRIC PEPSINOGEN OF: (a) TREATMENT OF INTACT AND ADRENALECTOMIZED RATS WITH ACTH, (b) HYPOPHYSECTOMY PLUS ADRENALECTOMY
For control and adrenalectomized values see Table 1

Type of experiment	No. of rats	Mean initial weight (gm.)	Mean weight loss (gm.) in 7 days	Mean stomach pepsinogen (P.U.)	S.D. ±	Compared to	
						Controls (Table 1): P	Adrenalectomized: P
ACTH-gel, 10 u/day:							
Intact	24	335	25	27,595	10,090	<.001	<.001
Adrenalectomized on salt water	10	319	62	4,520	1,884	<.001	<.02
Adrenalectomy and and hypophysectomy	7	252	122	4,570	1,110	<.001	<.05

TABLE 3. THE DEPRESSANT EFFECT OF MASSIVE DOSES OF CORTISONE ON RAT STOMACH PEPSINOGEN AND THE INFLUENCE OF STARVATION AND Na DEPLETION AND REPLETION

Type of experiment	No. of rats	Mean initial weight (gm.)	Mean weight loss (gm.) in 1 week	Mean pepsinogen (P.U./stomach)	S.D. ±	Compared to*		
						Controls (Table 1)	Adrenalectomized (a)	25 mg. Cortisone (a)
Adrenalectomized:								
(a) salt water full diet	27	328	14	10,377	6,910	<.001	—	<.01
(b) tap water full diet	13	318	44	8,053	3,890	<.001	>.3	<.05
(c) salt water starved	6	269	55	5,566	1,892	<.001	<.01	>0.5
25 mg. Cortisone per day:								
(a) Adrenalectomized + salt water	12	325	83	4,625	2,181	<.001	<.01	—
(b) Adrenalectomized + Na/K 2 mEq./day	10	320	77	10,770	5,041	<.05	>.05	<.01
(c) Adrenalectomized + K, 4 mEq./day	7	331	65	5,010	1,857	<.001	<0.1	>0.5
(d) Intact full diet	6	323	44	13,150	2,583	>0.2	>0.5	<.001
Intact								
(a) Starved (Pair fed with Cortisone (a))	4	316	27	13,600	1,480	>.2	>.2	<.01
(b) K depleted (2 weeks)	9	336	15	13,855	2,182	>.2	<.02	<.01
(c) Na depleted (2 weeks)	4	325	22	9,740	4,250	<0.1	>.5	<.02

* Significance of difference of means (Student's "t" test) values of P.

tomized rats were pair-fed with the group of adrenalectomized rats given 25 mg. cortisone per day, receiving 5.5 gm. food per day. The results in Table 3 show that this degree of starvation was insufficient of itself to reduce gastric pepsinogen in *intact* animals, but did cause a profound fall in gastric pepsinogen in the *adrenalectomized* rats.

In a group of 7 adrenalectomized rats the administration of potassium supplements (4mEq./day as KCl) by injection failed to alter the effects of the cortisone. In a further group of 10 rats, however, sodium and potassium (2mEq. of each per day as NaCl and KCl respectively) by injection did partly prevent the gastric effects without preventing the weight loss and debility induced by the cortisone. In 9 intact rats fed a synthetic diet of cornstarch, lard, and sugar providing less than 0.02 mEq. of potassium per rat per day for 2 weeks, there was no reduction of pepsinogen. In 4 other intact rats a synthetic sodium depleting diet (less than 0.04 mEq. sodium per rat per day) for 2 weeks did reduce the gastric pepsinogen, but not to levels significantly below the normal controls.

Four intact male rats and 2 pregnant adrenalectomized female rats at term showed neither the extreme debility nor the reduction in gastric pepsinogen when given a daily dose of 25 mg. cortisone.

Hypophysectomy (Table 2). Rats which had undergone hypophysectomy and bilateral adrenalectomy were found to have gastric pepsinogen levels significantly below both the intact controls and the adrenalectomized animals.

Gastric Erosion. Gastric erosions were found in none of the rats in any of the groups except:

1. Adrenalectomized rats given ACTH. Nine of 11 rats in this group had erosions in the glandular portion of the stomach, and many showed evidence of gross bleeding from the erosions.

2. Adrenalectomy plus hypophysectomy. Gastric erosions were present in 4 of 7 in this group, and all 4 bled from this source.

DISCUSSION

It has been shown that adrenalectomy decreases the pepsinogen content of the rat's stomach. That this effect is due to a reduction in the synthesis of the enzyme, rather than to increased secretion with decreased storage, is evident from the findings of others that the amount of pepsin secreted into the gastric juice is reduced by adrenalectomy (1, 5). The presence of adrenocortical steroid appears, therefore, to be necessary for the elaboration of normal amounts of pepsinogen by the gastric mucosa. This requirement for adrenal corticoids is evidently quite nonspecific, however, for normal levels of gastric pepsinogen were restored by a variety of different steroids, with widely differing glucocorticoid and mineralocorticoid potencies.

On the other hand, none of the individual steroids used produced an increase in gastric pepsinogen content above usual levels, such as was seen

with ACTH. This apparent discrepancy between the effects of ACTH and of the individual steroids may indicate that increased amounts of more than one steroid are needed to raise pepsinogen levels above normal. The failure of adrenal cortex extract to reproduce the stimulant effects of ACTH administration does not necessarily invalidate such a possibility, since the extract might elevate blood steroid levels intermittently (given, as it was, every 12 hours) as compared with the almost continuous elevation which might be expected from stimulation of the adrenal cortex by corticotropin gel. Furthermore, the dosage of extract may have been too small to increase gastric pepsinogen above normal. It is also possible that the different proportions of adrenal steroids contained in adrenal cortex extracts obtained from cattle, as compared with the endogenous adrenocortical secretions of the rat (3), may have influenced the results. The stimulant effect of ACTH on gastric pepsinogen cannot be attributed to a *direct* action of ACTH on the stomach or some other extra-adrenal site, since the action was abolished or even reversed by adrenalectomy.

The most marked reduction of gastric tissue pepsinogen was found in three groups of adrenalectomized rats. The first were hypophysectomized 1 week before, the second received 10 u ACTH gel/day, and the third received 25 mg. cortisone. In attempts to elucidate the mechanism of the effects of the large doses of cortisone, pair-feeding experiments showed that the reduction of gastric tissue pepsinogen could be reproduced by equivalent degrees of starvation in adrenalectomized but not in intact rats. Partial prevention of the effects of cortisone by supplementation with NaCl and KCl (but not by KCl alone), however, despite an almost equal degree of debility (77 vs. 83 gm. mean weight loss) indicates that anorexia and weight loss alone may not be the sole explanation of this striking effect of cortisone in massive doses on gastric pepsinogen. The observations suggest that the great reduction of pepsinogen seen in these groups may be produced by sodium depletion resulting from starvation in the presence of adrenalectomy. It is also possible that the large doses of cortisone used may have induced sodium depletion partly by a natriuretic effect similar to that exerted by hydrocortisone in smaller doses (6). The reduction of gastric pepsinogen in intact rats by sodium depletion further supports a role of sodium deficiency in this phenomenon.

As an incidental observation, gastric ulceration was observed frequently, and only, in adrenalectomized rats receiving ACTH, or subjected to hypophysectomy. While the significance of these observations is not understood, they do indicate that peptic ulceration may occur in rats in the absence of adrenocortical steroid administration or the endogenous liberation of such steroids.

SUMMARY

The influence of the adrenal cortex was studied on gastric tissue pepsinogen content, measured directly on homogenized rat stomachs.

Adrenalectomy significantly reduced gastric tissue pepsinogen below normal control values. Individual steroids (cortisone, corticosterone, hydrocortisone, DCA) and adrenal cortex extract were administered to adrenalectomized rats. While in a certain dose each restored tissue pepsinogen to normal, none produced an increase above normal. By contrast, ACTH given to intact rats significantly increased gastric pepsinogen.

A significant further reduction below the adrenalectomy levels was observed in adrenalectomized animals which were also hypophysectomized or which were also given 10 u ACTH or 25 mg. cortisone daily or which were starved. From various experiments made to modify the depressant effects of these massive doses of cortisone, it was concluded that the combination of adrenalectomy and starvation was necessary for the greatest reductions in gastric tissue pepsinogen, and that these reductions were probably attributable to sodium depletion in the face of adrenalectomy.

Gastric mucosal ulceration was observed in the adrenalectomized rats which were also hypophysectomized or which received ACTH.

Acknowledgment

We wish to thank Dr. C. J. O'Donovan of The Upjohn Company, Kalamazoo, Michigan, for generous supplies of adrenal cortex extract and corticosterone.

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THE RECOVERY OF 3:5:3′-TRIIODOTHYROACETIC ACID AND 3:3′-DIIODOTHYRONINE FROM RAT KIDNEY AFTER INJECTION OF 3:5:3′-TRIIODOTHYRONINE[1]

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THE four thyroid hormones actually known, L-3:5:3′:5′-tetraiodothyronine or thyroxine, L-3:5:3′- and L-3:3′:5′-triiodothyronine and L-3:3′-diiodothyronine, are metabolized according to two principal ways: dehalogenation and oxidative deamination. This leads to the iodothyropyruvic acids, present in bile and urine (1) and the question of their decarboxylation in the iodothyroacetic homologues remains open (2). The metabolic formation of the iodothyroacetic acids from the iodothyronines would be of interest, because of the biological properties of the former. These exert an immediate action on oxygen consumption of rats (3), of liver and kidney slices (4) and on glycolysis of ascites tumor cells *in vitro* (5), in experimental conditions where thyroid hormones are effective only after a more or less important lag of time or are ineffective.

The scope of our research was to detect the eventual presence of 3:5:3′-triiodothyroacetic acid (TRITA) and of 3:3′-diiodothyronine (DITh) in the kidney of rats injected with labelled L-3:5:3′-triiodothyronine (TRITh). After having shown TRITA to be present in rat kidney, we studied some aspects of its metabolism in thyroidectomized animals.

MATERIALS AND METHODS

I. *Preparation of minute amounts of labelled TRITh and of TRITA of high specific activity*

TRITh was synthesized by iodination on 3′ of 3:5-diiodothyronine, according to a technique previously described (6). The solution of labelled I_2 was prepared by exchange between I^{127} and I^{131}. The aqueous I^{131} solution, of a radioactivity of 2 mc., was placed in an elbow micropipette and evaporated in a desiccator. The dry residue was taken up with 5 μl. of 0.005 N H_2SO_4 to bring the pH close to 5. Separately, a solution of I^{127} was prepared, containing 86 mg. I_2 in 50 ml. ethanol. Seven μl. of this was taken up in a

Received April 12, 1956.

[1] The following abbreviations have been used throughout the text:

DITh = 3:3′-diiodothyronine
TRITh = 3:5:3′-triiodothyronine
TRITA = 3:5:3′-triiodothyroacetic acid
I^- = Iodide

[2] With the technical assistance of Mrs. D. Cousseran (CNRS).

graduated micropipette and added to the solution of I^{131}. The substitution reactions were carried out immediately after the exchange between I^{127} and I^{131}; the iodination of 3:5-diiodothyronine was carried out in a hematological micropipette by first taking up in its bulb 14 μl. of a solution of 3:5-diiodothyronine (2.86 mg. of DITh in 2 ml. of concentrated NH_4OH of d = 0.89) and then by sucking in all of the radioactive iodine solution. The liquid was well mixed and left to react for about 15 minutes.

The relatively abundant iodide was separated by paper ionophoresis of the reaction mixture by placing it on a sheet of Whatman paper No. 1, 35 cm. × 17 cm. The separation of the radioactive iodide was carried out in 0.2$M(NH_4)_2CO_3$ buffer (pH 9.5) at 180 volts and 13 mA (150 minutes). The paper was then dried and the position of the different radioactive products revealed by radioautography. Under these conditions, 3:5:3′-triiodothyronine does not move, while the iodides are found at about 9 cm. from the starting line. The strip of Whatman paper corresponding to the iodothyronines was cut out and the products of synthesis eluted by water-saturated *n*-butanol. The eluate was subjected to chromatographic analysis in order to isolate the 3:5:3′-triiodothyronine. Chromatography was carried out in ter-pentanol saturated with 2N NH_4OH (R_f TRITh, 0.46) and the TRITh eluted by water-saturated *n*-butanol. The eluate was subjected to chromatographic analysis in order to isolate the 3:5:3′-triiodothyronine. Chromatography was carried out in ter-pentanol saturated with 2N NH_4OH (R_f TRITh, 0.46) and the TRITh eluted by water-saturated *n*-butanol. The eluate was then concentrated under reduced pressure under N_2 and the dry residue taken up by 2 ml. of water and brought to pH 7.5 with 1N NaOH. The specific activity of the 3:5:3′-TRITh obtained was 200 μc./μg.

TRITA was synthesized by iodination of 3:5-diiodothyroacetic acid in position 3′ in accordance with the procedure previously described. The labelled iodine solution was prepared by an exchange reaction between a solution of I^{131} containing 3.14 mc. evaporated to dryness, and 7 μl. of an I^{127} solution containing 67 mg. I_2 in 100 ml. of 95% ethanol.

The iodination reaction was carried out in the hematological micropipette and the radioactive iodide separated by ionophoresis on Whatman No. 1 paper (0.2 M $(NH_4)_2CO_3$ buffer, 180 volts and 13 mA for 150 min.).

The iodothyroacetic acids were then eluted with water-saturated *n*-butanol and the eluate subjected to another ionophoresis to separate 3:5:3′-tri- and 3:5:3′:5′-tetraiodothyroacetic acids (Whatman No. 1 paper, 40 × 20 cm., 0.05 M $(NH_4)_2CO_3$ buffer, 5 hrs. at 480 v. and 16 mA).

The mobilities of 3:5:3′-tri- and 3:5:3′:5′-tetraiodothyroacetic acids were then respectively 5 and 7 cms. The strip corresponding to 3:5:3′-triiodothyroacetic acid was cut out, eluted with water-saturated *n*-butanol, and the eluate distilled under reduced pressure and under N_2. The dry residue was taken up by 2 ml. of water and brought to pH = 7.5 with 1N NaOH. The specific activity of the 3:5:3′-triiodothyroacetic acid obtained was 80 μc. per μg.

II. *The Identification of 3:3′-DITh and of TRITA*

Separation and identification of these two derivatives have been obtained by paper chromatography and by paper electrophoresis, under very definite conditions. The chromatographic behavior of a few iodothyronines and iodothyroacetic acids has been studied in two solvents: *n*-butanol saturated with 2N NH_4OH. Values of R_f (Whatman paper No. 1, t° = 17°) are recorded in Table 1.

The electrophoretic mobility of these substances has been studied on Whatman paper No. 1, in 0.05 M ammonium carbonate buffer at pH 9.0, with 12 V/cm. length of paper and 1 mA/cm. width, and development of the spots using the Pauly reaction. Under these conditions, thyroxine and 3:5:3′-triiodothyronine do not move, while 3:3′-diiodothyronine is found at 3.50 cm. from the starting point, the 3:5:3′-triiodothyro-

TABLE 1. R_f VALUES OF THE VARIOUS IODINATED DERIVATIVES

Substances	R_f in *n*-butanol saturated by 2N NH_4OH (t°17°)	R_f in tertiary pentanol saturated by 2 N NH_4OH (t°17°)
Thyroxine (Tx)	0.48	0.28
3:5:3′-triiodothyronine (TRITh)	0.65	0.46
3:3′-diiodothyronine (DITh)	0.51	0.36
3:5:3′:5-tetraiodothyroacetic acid (TETRATA)	0.66	0.40
3:5:3′-triiodothyroacetic acid (TRITA)	0.75	0.55
Iodide	0.31	0.18

acetic acid at 6.0 cm. and the 3:5:3′:5′-tetraiodothyroacetic at 7.5 cm. Identification of 3:5:3′-tri- and 3:5:3′:5′-tetraiodothyroacetic acids is thus possible in the presence of thyroxine, 3:5:3′-triiodothyronine and 3:3′-diiodothyronine.

METABOLIC DERIVATIVES OF L-3:5:3′-TRIIODOTHYRONINE IN RAT KIDNEY

Groups of three male rats (180 gm). thyroidectomized 15 days previously were given, by intraperitoneal injection, 0.25 μg./rat of TRITh labelled in position 3′ by 50 μc. I^{131} (6) and killed four hours later. The kidneys (3.75 gm.) were immediately frozen, ground with dry ice to a fine powder, and taken up with 25 ml. of 1% NH_4OH. The renal extracts contained about 2% of the radioactivity injected; they were lyophilized, and the product obtained was extracted five times with 10 ml. of an ether-ethanol mixture (95% peroxide-free ether and 5% absolute ethanol). Less than 1% of the radioactivity went into the ether layer. The dry lipid-free residue was taken up with 2 ml. of water, then dissolved by careful addition of 1 ml. 2N NH_4OH. The proteins were precipitated with 7.5 ml. of 95% ethanol and eliminated by centrifugation. The supernatant was separated and a second extraction carried out on the residue under the same conditions.

To the ammoniacal solution (18 ml.) containing 85% of the radioactivity present in the kidneys 18 ml. of $CHCL_3$ were added, then 6 ml. of 2N NH_4OH. After centrifugation, the upper layer was discarded and the extraction repeated twice with 6 ml. of 2N NH_4OH. Eighty per cent of the initial radioactivity was recovered. All operations were carried out at 0° C and under an atmosphere of CO_2, to avoid any deiodination.

The ammoniacal extracts were evaporated under reduced pressure under N_2 to about 2 ml., and the concentrated solution obtained was placed on Whatman paper No. 1 and eluted with water-saturated *n*-butanol. The pigments remained on the paper and the eluate contained nearly all of the radioactivity. A sample was analyzed by bi-dimensional chromatography (*n*-butanol saturated with 2N NH_4OH and ter-pentanol saturated with 2N NH_4OH). The radioautogram showed four main spots and a few fainter ones.

The positions of these four spots are identical with those of reference spots of 3:5:3′-TRITA, 3:5:3′-TRITh, 3:3′-DITh and iodide I^-.

The radioactive spots found on the radioautogram were cut out and their

radioactivity measured in the well-type scintillation counter which enabled us to determine quantitatively the radioactivity among the various renal metabolites of 3:5:3′-triiodothyronine. The results obtained (% of total radioactivity) are as follows: 78% as in 3:5:3′-triiodothyronine, 6.4% as in 3:3′-diiodothyronine, 9.7% as in 3:5:3′-triiodothyroacetic acid, 2% as in iodide and 4% as in the unknown substances.

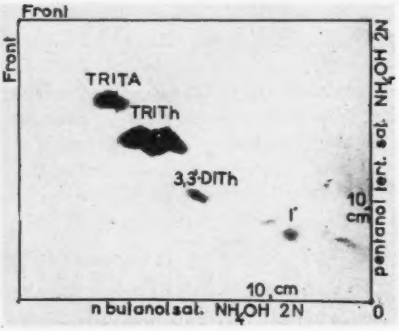

FIG. 1. Bidimensional chromatogram of renal extract of rats treated with 0.25 μg. TRITh.
I⁻ = iodide.
DITh = 3:3′-diiodothyronine.
TRITh = 3:5:3′-triiodothyronine.
TRITA = 3:5:3′-triiodothyroacetic acid.
Solvents: *n*-butanol saturated with 2N NH_4OH and ter-pentanol saturated with 2N NH_4OH.

In order to confirm the presence of 3:5:3′-TRITA the remaining total extract was purified by preparative chromatography in *n*-butanol saturated by 2N NH_4OH. After development, two strips of R_f 0.65 (TRITh) and 0.75 (TRITA) were cut out on the chromatogram and eluted using water-saturated *n*-butanol. The two eluates were separately analyzed by unidimensional chromatography (solvent ter-pentanol saturated with 2N NH_4OH) followed by electrophoresis at right angles, for 5 hours, in 0.05 *M* $(NH_4)_2CO_3$ buffer, pH 9.0 (12 V/cm. length and 1 mA/cm. width).

The radiochromatoelectrophoregram of the eluate of the strip at the level of TRITh (Fig. 2) shows two radioactive spots, one of TRITh (R_f 0.46), which did not move under electrophoresis, the other, weaker, of TRITA (R_f 0.55), which moved 6.25 cm. The strip at the level of TRITA has only one spot, matching the TRITA reference spot (R_f 0.55) and which moved 6.25 cm.

From all of these results the presence of 3:3′-diiodothyronine and 3:5:3′-triiodothyroacetic acid has been established in rat kidney following injection of physiological doses of L-3:5:3′-triiodothyronine.

METABOLISM OF 3:5:3′-TRIIODOTHYROACETIC ACID IN RATS

I. *The distribution of 3:5:3′-triiodothyroacetic acid in various organs*

We have given physiological doses of 3:5:3′-triiodothyroacetic acid labelled in 3′ by I^{131} to several groups of male rats weighing about 180 gm., thyroidectomized at least three weeks before and kept all this time at a temperature of 25°–1° C. Each rat was injected intraperitoneally with 0.5 ml. of a solution of 0.075 μg. 3:5:3′-triiodothyroacetic acid labelled by 6.20 μc. of I^{131}, at pH = 8. The animals were killed 30, 60 and 180 minutes after

the injection. The brain, the heart, the liver, the intestinal tract, the stomach, the kidneys, the adrenals, some muscle and blood were collected. The radioactivity per gram of tissue was measured with the windowless gas counter on aliquots of organ tissue which had been liquefied by adding 0.5 ml. of 1N NaOH, boiled and diluted to a volume of 3 ml. and then desiccated. For the adrenals, the radioactivity was measured after liquefaction

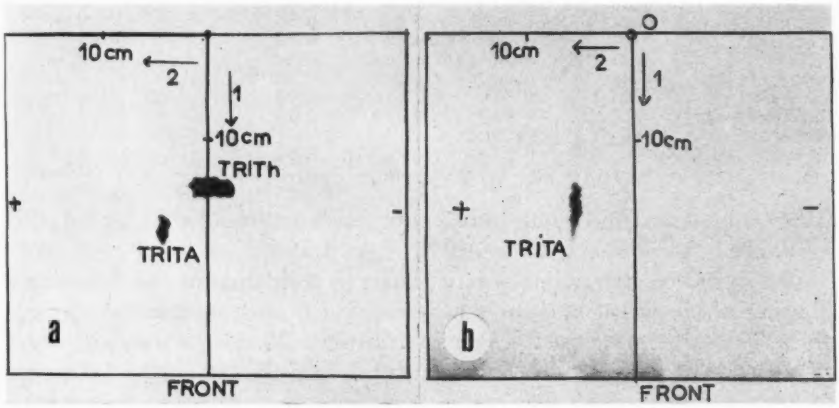

FIG. 2. Chromatoelectrophoregram a) of the strip at the level of TRITh; b) of the strip at the level of TRITA.

1st dimension: ter-pentanol saturated with 2N NH_4OH.

2nd dimension: electrophoresis in 0.05M $(NH_4)_2\ CO_3$ buffer, pH = 9.

of the whole glands. The results obtained expressed in per cent of radioactivity injected (brought to the initial value found in the entire organ) and per gm. of organ are given in Table 2.

Most of the radioactivity found in the organs was present in the kidney, liver and the digestive tract thirty minutes after injection. Later there was a definite decrease of I^{131} in the intestine; however, it remained practically constant in the liver and the kidneys, even after three hours. The opposite was true of the thyroid hormones, the rapid decrease of which is a result of their intense entero-hepatic circulation (7).

The concentration per gm. of organ never reached a very high value as compared to that in the blood. However, it can be considered that there was a definite accumulation in the kidneys. There was an early concentration in the adrenals, which later lost the major part of their radioactivity.

II. *Urinary and fecal elimination of I^{131} after injection of 3:5:3'-triiodothyroacetic acid*

Each animal of a group of 5 male rats weighing about 170 gm., thyroidectomized more than three weeks earlier and kept all this time at a temperature of 25° ± 1° C received by intramuscular injection 0.5 ml. of a solution,

TABLE 2. DISTRIBUTION OF 3:5:3′-TRIIODOTHYROACETIC ACID IN VARIOUS ORGANS AFTER INJECTION

Organ	%1131 injected recovered after:					
	30 minutes		1 hour		3 hours	
	(in the whole organ)	(per gm. of organ)	(in the whole organ)	(per gm. of organ)	(in the whole organ)	(per gm. of organ)
Brain	0.08	0.02	0.07	0.016	0.025	0.007
Heart	0.14	0.08	0.09	0.09	0.08	0.04
Stomach	0.50	0.24	0.24	0.10	0.51	0.15
Liver	3.33	0.20	3.14	0.16	2.97	0.12
Kidney	1.80	0.40	1.26	0.26	1.30	0.22
Adrenals	0.05	0.51	0.012	0.13	0.009	0.06
Intestinal tract	3.75	0.25	1.13	0.14	0.79	0.14
Muscle	—	0.03	—	—	—	0.02
Plasma	—	0.21	—	0.07	—	0.10

at pH = 8, of 3:5:3′-triiodothyroacetic acid containing 0.05 μg. of this substance labelled in 3′ by 5 μc. of I^{131}.

After injection, the animals were placed in individual metabolism cages allowing a separation of urine and feces, which were sampled at various times. The urines were used as such for the determination of the radioactivity with the windowless gas counter, after drying on plates. The feces were air dried and pulverized. One gm. of homogeneous powder was boiled for 5 to 20 minutes with 1 ml. of 1 N NaOH. The volume was brought to 5 ml. with distilled water and an aliquot was taken out to measure the radioactivity with the gas counter. Figure 3 shows the details on the elimination of I^{131}.

The rate and intensity of urinary and fecal elimination of TRITA wer comparable to those of 3:5:3′-TRITh (8). The former was not metabolized more rapidly than the latter, since only 38% of the injected radioactivity was found in the excreta after 96 hours.

DISCUSSION

A small portion of I^{131} injected into thyroidectomized rats (about 2%) as L-3:5:3′-triiodothyronine labelled in 3′ was found in kidneys after four hours. The chromatographic analysis of the tissue extracts shows the presence of the injected hormone and three of its derivatives: iodide, 3:3′-diiodothyronine and 3:5:3′-triiodothyroacetic acid (in all, 96% of extracted radioactivity).

The presence of 3:3′-DITh is probably due to a local dehalogenation because the catabolism of this substance has been previously shown as proceeding at a high rate. 3:5:3′-TRITA can be formed in kidney or concentrated by it. In any case, it is apparently produced by oxidative decarboxylation of 3:5:3′-triiodothyropyruvic acid which was derived from the oxidative deamination of 3:5:3′-TRITh. The keto-acid has been shown to be present in bile and urine after injection of 3:5:3′-TRITh.

The presence of 3:3′-DITh and iodide is a definitive proof of the stepwise deiodination of 3:5:3′-TRITh and is an indirect proof of an eventual

formation of the latter from thyroxine, as shown by Albright, Larson and Tust (9) in their experiments on kidney. Anyhow, it may be suggested that the formation of the 3:3′-derivative, also found in other organs, is of special significance, not only because of its very high rate of diffusion (11), but also because it can, eventually, be destroyed by oxidases effective on thyronine and on 3′-monoiodothyronine, but not on thyroxine and the triiodothyronines (11).

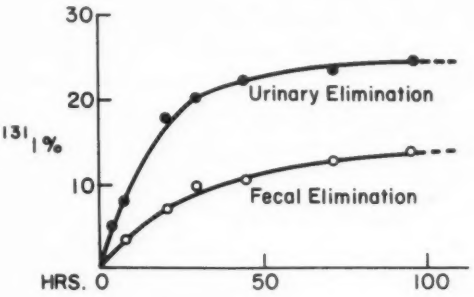

FIG. 3. Elimination of I^{131} in urine and feces of thyroidectomized rats, after intramuscular injection of 0.05 μg. 3:5:3′-triiodothyroacetic acid labelled on 3′.

The biological formation of 3:5:3′-triiodothyroacetic acid in intact animals has been shown for the first time in our researches. The eventual importance of this substance as an active hormone derivative is therefore strengthened. It must be pointed out that it appears in a relatively short time after the injection of its hormonal precursor. In any case, its presence in kidney is significant as indicating one metabolic pathway of degradation of the iodothyronines. The catabolism of these includes apparently two main processes: stepwise deiodination and oxidation of the alanine residue with successive transformation of a pyruvic to an acetic residue.

The distribution of 3:5:3′-TRITA shows a difference with that of 3:5:3′-TRITh. Its concentration by the liver is greater and more rapid at the beginning (16% in one hour), but it goes down fairly rapidly subsequently; that of 3:5:3′-TRITA is definitely less but more durable. Such differences may mean the absence of an entero-hepatic circulation of TRITA, while that of 3:5:3′-TRITh is well established. However, the urinary and fecal elimination of 3:5:3′- TRITA shows the same characteristics of that of its precursor. Yet, in general, the metabolic behavior of the two products seems fairly close. Then, if the tissue effect of TRITA is immediate while it appears with 3:5:3′-TRITh only after a lag of time, it is probable that the mode of action of the thyroid hormones is not entirely dependent on metabolic transformations of their carbon skeleton. Furthermore, the partial deiodination of the hormones, one aspect of which is revealed by the transformation of 3:5:3′-TRITh to 3:3′-DITh, greatly changes their activity.

SUMMARY

Two metabolic derivatives of L-3:5:3′-triiodothyronine, namely 3:3′-diiodothyronine and 3:5:3′-triiodothyroacetic acid have been shown to be

present in the rat kidney after administration of the first compound labelled with I^{131} on 3′. The presence of L-3:3′-diiodothyronine shows the partial deiodination of the triiodinated hormone, and that of 3:5:3′-TRITA proves the oxidative degradation of the alanine residue of the latter (oxidative deamination, followed by decarboxylation of the iodothyropyruvic acid formed).

The metabolism of thyroid hormones may take two pathways, deiodination followed by rupture of the phenoxy bond, with or without oxidative degradation of the alanine residue.

The distribution in tissues of 3:5:3′-triiodothyroacetic acid injected in physiological quantities and its urinary and fecal elimination are in many ways similar to those of L-3:5:3′-triiodothyronine.

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ALLOXAN DIABETES IN THE HAMSTER: ORGAN CHANGES DURING THE FIRST DAY[1]

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INTRODUCTION

GAARENSTROOM and Siderius (10) in the symposium, "Experimental Diabetes," discussed several points which bear directly on our investigation of alloxan diabetes in the hamster. First, they indicate that there is no direct evidence of the causation of the hypoglycemic phase by release of insulin from damaged islets. Second, they suggest that the hypo phase results from the exhaustion of liver glycogen during primary hyperglycemia. Next, concerning the theory that alloxan is primarily stimulatory, they state "A further indication that alloxan stimulates the pancreatic islets during the first hours would be the occurrence of histologically recognizable activity of islet cells." Lastly, they emphasize that most investigators report only degenerative lesions from observations made several hours after treatment.

Due to the striking granulation pattern in normal beta-cells, the hamster pancreas is well-adapted to the study of all of these problems. Since liver and kidney also show marked changes, heretofore unreported, we have included these as well.

METHODS

Over 200 hamsters, evenly divided as to sex, were used. Their ages varied between 3 months and one year, and their weights ranged from 80 to 130 gm. Animals were paired according to size and sex, and maintained on Purina Laboratory Chow. After weighing, groups of 16 to 18 were placed under anesthesia by the intraperitoneal injection of Nembutal.[4] At this time, one or two animals, typical of the group, were sacrificed to obtain normal tissues. The others were injected intravenously through the ocular sinus by the method used in previous experiments (19). A freshly-prepared 3% solution of Eastman alloxan was given at the rate of 55–60 mg./kg. At intervals of 5 minutes to 24 hours, a 0.10 ml. blood sample was withdrawn from the sinus through an heparinized

Received May 8, 1956.

[1] Supported in part by a grant from the Council on Pharmacy and Experimental Therapeutics of the American Medical Association.

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[4] Lackey *et al.* (16) established that this type of anesthetic does not affect tissue glycogen.

needle and analyzed for sugar according to the anthrone technic of Durham (8). As soon as the blood was taken, the animal was sacrificed and small pieces of pancreas, liver and kidney were fixed in Bouin's fluid and in picric-acid-absolute-alcohol glycogen fixative. Tissues were treated with cellosolve, benzene, 60° paraffin and were sectioned at 5 and 7 micra. Sections were stained by our modification of Gomori's (13) aldehyde fuchsin[5] and by Lagerstedt's (17) Gallocyanin-Chrome-Alum. Glycogen-fixed tissues were stained by periodic acid-Schiff method (18). Adjacent sections were digested in 0.10 malt diastase buffered to pH 6.5, followed by gallocyanin or Schiff staining. Sections from experimental animals were then compared to similar sections from controls. Both hepatic nucleic acids, as demonstrated by gallocyanin, and glycogen were estimated in terms of optical density, on an arbitrary scale of 0 to 4, with higher numbers representing greater concentration.

OBSERVATIONS

This report will confine itself to the most severe type of alloxan reaction, involving the degeneration of 95% of the beta-cells. The transformations occurring within the first day have been divided into four processes, based upon the characteristic changes in larger islets. It should be pointed out that the time limits for each are highly variable, the rate of drug effect showing much individuality.

1. *Hypergranulation* (First 40 minutes)

Within 5 min., beta-cells adjoining sinusoids have completely filled with granules (Figs. 2, 3). In 15 min. all beta-cells are full. At the same time, GCA[6] sections indicate a definite increase in nuclear and cytoplasmic basophilia (Figs. 4, 5). Granule concentration reaches its peak in about 40 min. A slight elevation of blood sugar occurs in this period (Fig. 1).

2. *Areolation* (45 min. to 2 hrs.)

Forty-five min. after alloxan, spaces appear around the sinusoids. These become progressively more pronounced (Fig. 6). Within 1 hr., small clefts appear among some of the cells (Fig. 6). Areolation is most pronounced after 2 hrs. During this phase, less than 5% of beta-cells show degranulation or degeneration. GCA sections show no additional alterations. Blood sugar levels rise to the first hyperglycemic crest during this time.

3. *Degeneration*

a. *Early* (2–5 hrs.)

The onset is characterized by the narrowing of the recently-formed intercellular spaces. This condition is evident 2½ hrs. post alloxan, and continues until all clefts have been obliterated and the beta-cells have fused together into a solid, granular mass, containing scattered nuclei. The latter are becoming smaller, darker, ovoid and more homogeneous. Coales-

[5] The authors wish to acknowledge with thanks the generous technical assistance rendered by Grace M. House.

[6] This is an abbreviation for gallocyanin-chrome-alum.

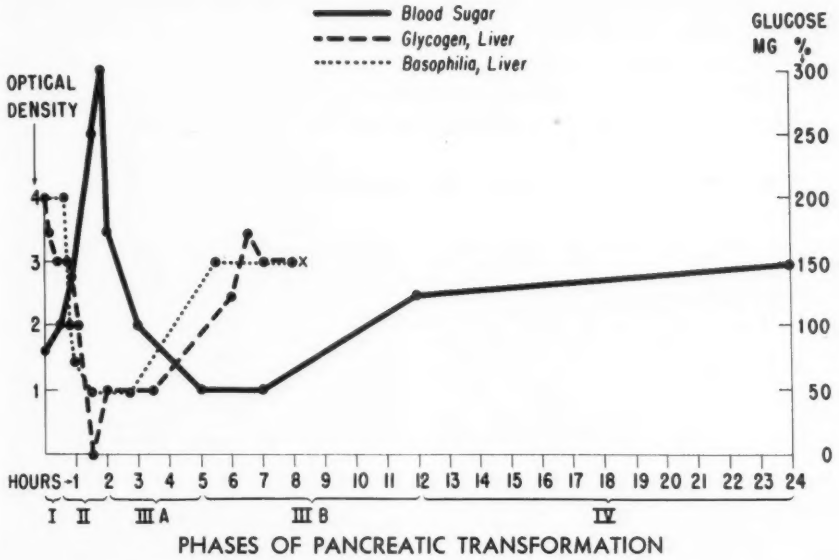

FIG. 1. The relationship between blood sugar, liver glycogen and liver basophilia (RNA) is shown. The latter are reported in terms of optical density, numbers 0–4, on the left. Blood sugar, in mg% appears on the right. From point "X," the lines representing glycogen and basophilia show much individuality but have a tendency to fluctuate in an intermediate zone between maximum and minimum values. The phases of pancreatic transformation are indicated beneath the hours.

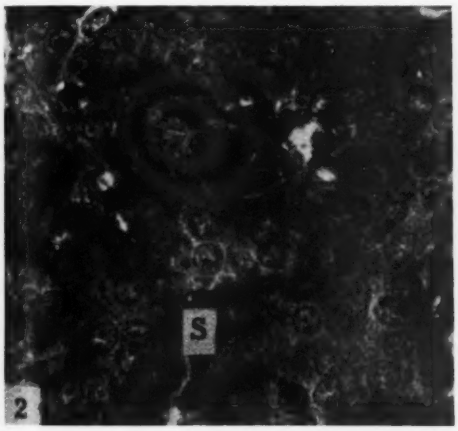

FIG. 2. Normal islet. Note the distribution of granules along sinusoid "S." Aldehyde fuchsin. ×420.

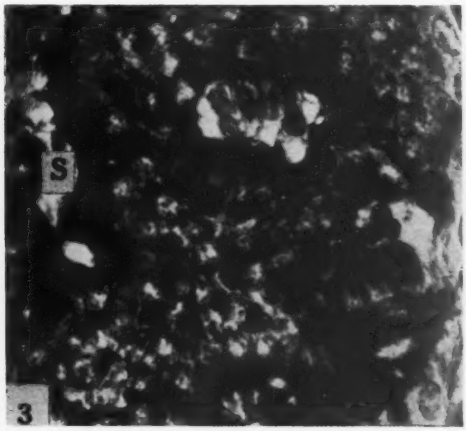

FIG. 3. Islet 5 minutes after alloxan showing an increase in number of granules. Aldehyde fuchsin. ×420.

cence is complete in 5 hrs. (Fig. 7). The GCA series shows a concentration of basophilic substance in obviously smaller nuclei accompanied by a gradual loss, complete in 5 hrs., of color from the cytoplasm (Fig. 8). The centrally-located "beta-mass" shrinks away from the peripheral ring of alpha-cells. Blood sugar values begin a rapid decline in this period.

b. *Late* (5–12 hrs.)

Soon after the appearance of the "beta-mass," small patches, usually

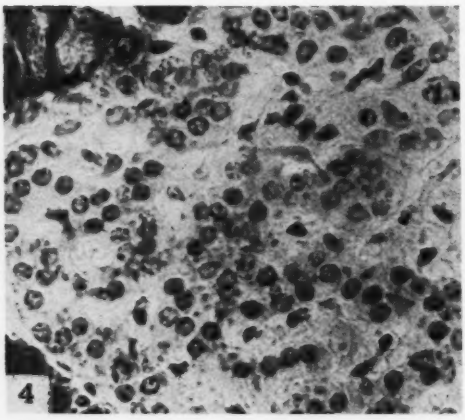

FIG. 4. Normal islet from same animal as Figure 2. The elongate dark nuclei outline the sinusoid. GCA. ×420.

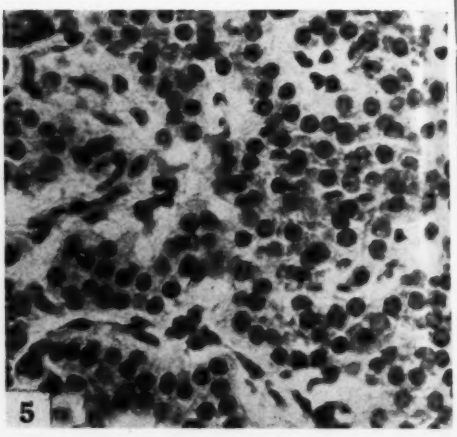

FIG. 5. Islet from same animal as Figure 3 showing an increase in density of both nuclei and cytoplasm. GCA. ×420.

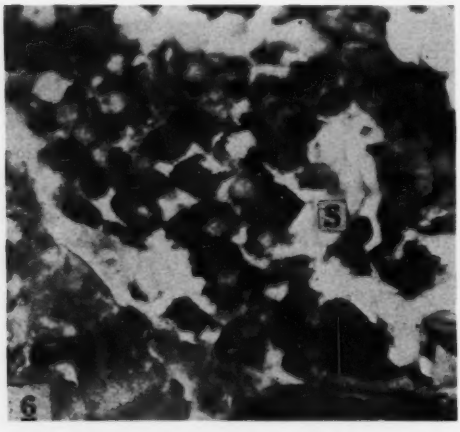

FIG. 6. Large islet 105 minutes after alloxan showing a space between the wall of a sinusoid "S" and the adjoining cells. Intercellular spaces are also visible. Aldehyde fuchsin. ×420.

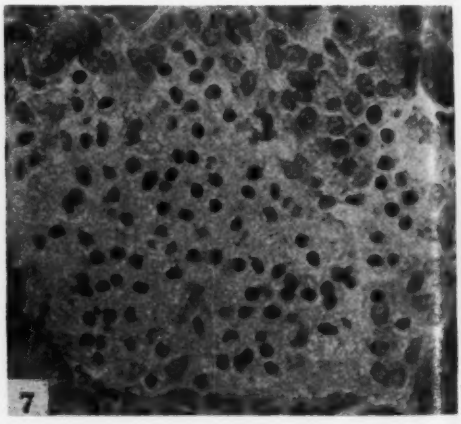

FIG. 7. Islet 5½ hours after alloxan showing shrinking and darkening of nuclei and loss of cytoplasmic density in cells near the center. Normal cells at upper right. Alpha-cell nuclei are oval. GCA. ×420.

in the area containing a nucleus, show changes in both color and granule distribution. First, adjacent granules clump, with clumps separated by minute crevices. At the same time there is a shift in color from purple to gray. The nuclei disintegrate and shortly only scattered debris remains. These areas become more numerous with the subsequent blending of foci of degeneration (Fig. 7). Finally, only a few spots resembling normal conditions can be found. Occasionally, deterioration is well-advanced 12 hrs. after treatment. After 24 hrs., the only evidence suggestive of beta-cells consists of small patches of a purplish, homogeneous substance (Fig. 9). The GCA series show a progressive disintegration of nuclei. The low in hypoglycemia occurs near the beginning of "late degeneration" while near its end, blood glucose values begin their second ascent.

4. *Substitution.* (12–24 hrs.)

Substitution begins long before disintegration ends. In areas adjoining sinusoids, an increase in fine, fibrous material (Fig. 7) is seen. A little later, cells having large, pale and extremely irregular nuclei, together with a colorless, agranular cytoplasm, appear from the perivascular tissue and the reticular islet "capsule." The centers of many islets are filled with such cells 18 hrs. after treatment. By 24 hrs. nearly all are in this condition. The GCA series show certain transformations among these bizarre cells, namely: nuclear outlines become more regular as the final bean or oval shape is achieved and nuclear volume decreases as basophilia increases (Fig. 10).

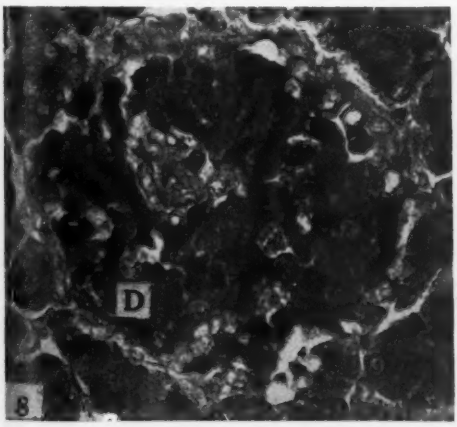

FIG. 8. Islet from same animal as above showing the coalescence of beta-cells. To the left and above the center connective tissue appears around a sinusoid. The pale patch above "D" is a focus of degeneration. Aldehyde fuchsin. ×420.

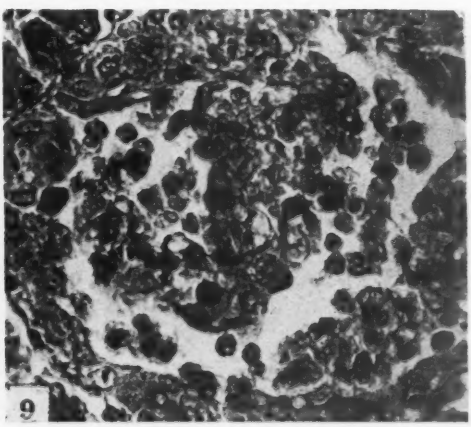

FIG. 9. Islet 24 hours after alloxan showing distintegration of cells near the center together with some of the substitute cells. Three dark spots near middle and extreme left are small, homogeneous beta masses. Aldehyde fuchsin. ×420.

Liver

Control animals showed hepatic parenchyma cells well-filled with glycogen (Fig. 11) and basophilic granules. Animals sacrificed 1 to 4 hrs. after alloxan injections exhibited depletion of both components, with the cytoplasm generally free of granules (Fig. 12). Those sacrificed earlier than

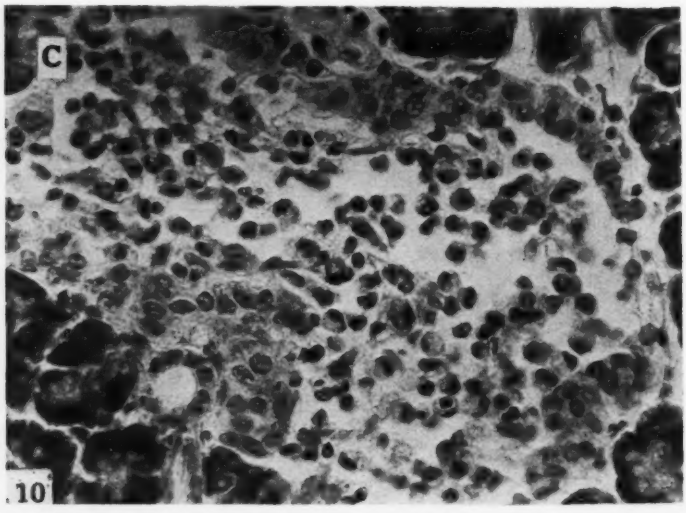

FIG. 10. Islet 24 hours after alloxan, same animal as Figure 9. Nuclei of various shapes are demonstrated. Near the periphery to the right of "C" substitution cells are differentiating. GCA. ×420.

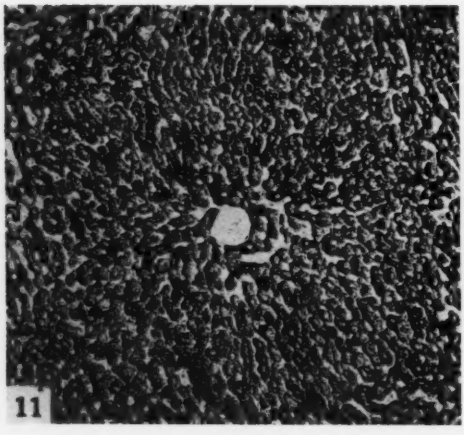

FIG. 11. Central portion of liver lobule, normal glycogen. PAS. ×120

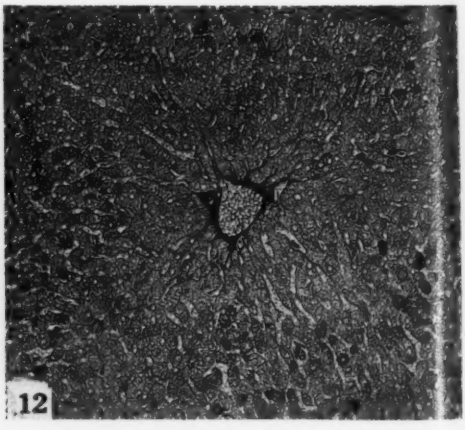

FIG. 12. Comparable section of liver from an animal in primary hyperglycemia. Depleted glycogen. PAS. ×120.

1 hr. or later than 4 hrs. had livers intermediate in appearance between the intact condition and the depleted condition. Animals allowed to survive 18 to 24 hrs. most nearly resembled the controls.

Depletion was found in two different patterns. In some animals, the periportal zones were most markedly agranular, with the central cones more nearly normal. In others, depleted cells were found in all zones, scattered among granulated cells.

Foci of small, basophilic cells, resembling lymphocytes, were common in the livers of the two series of animals. The sinusoidal phagocytes often appeared large and prominent.

Kidney

The kidney shows wide individual variations in its response to alloxan. Certain features, however, are general enough to warrant brief comment.

During the first 5 min., there is a reaction indicative of greatly increased arterial pressure within the kidney. Up to 60% of the glomeruli become distended with tremendous increases in the capsular spaces (Fig. 13). The capillaries themselves are distended and turgid (Fig. 14).

All parts of the nephron, including the convoluted tubules (Fig. 15) and Henle's loop are severely dilated.

At the same time 50% of the proximal tubules show concentrations of granules, of yet undertermined composition, and a lesser percentage contain a strange network of floccular substance (Figs. 16, 17).

Extreme tubular dilatation is characteristic of the first hr. and, to a lesser degree, of the first 12 hrs., diminishing toward the normal by the

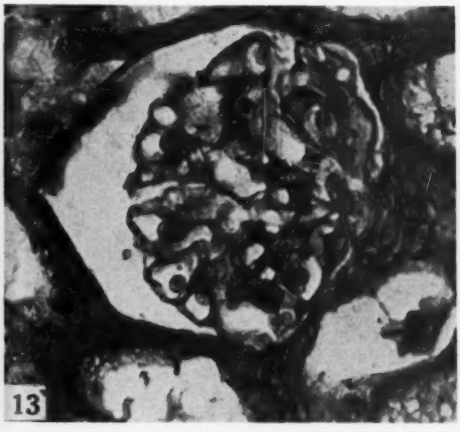

FIG. 13. Kidney cortex 5 minutes post alloxan showing distension of capsule and glomerular vessels. Aldehyde fuchsin. ×420.

FIG. 14. Higher magnification of the same kidney showing distension of capsule, engorgement of vessels and a granular substance in a proximal tubule, lower right. Aldehyde fuchsin. ×600.

end of the first day. Tubular granulation dropped markedly after 15 min. and was rare after ½ hr.

DISCUSSION

Although our first fig. appears to indicate a rise in blood sugar concomitant with hypergranulation, a relationship inconsistent with hyperinsulinism, a graph plotting sugar values against minutes during the first hr. shows that there is little significant rise for 25 or 30 min. A few animals actually show an early decline in blood sugar, following a tetraphasic curve similar to that of Shipley and Beyer (22) and others. This is suggestive of an increase in circulating insulin.

Toward the end of "hypergranulation" and throughout "areolation" blood sugar rises precipitously. There are several possible explanations for this incongruity. First, hypergranulation may not necessarily indicate insulin, per se, but may represent either a materialization of its precursors or some intermediate substance formed during its synthesis. Another suggestion involves the possibility that insulin is being formed more rapidly than it is being released. In either case, however, to account for hyperglycemia, an increased mobilization of carbohydrate would have to be assumed. This assumption is consistent with the observation that demonstrable glycogen almost completely disappears from the liver during the initial rise. The suggestion that hypergranulation may arise through accumulation of insulin in the cell, due to blockade of the insulin release mechanism by alloxan, is not supported by the observed engorgement of the islet sinusoids or by the marked increase of cytoplasmic basophilia.

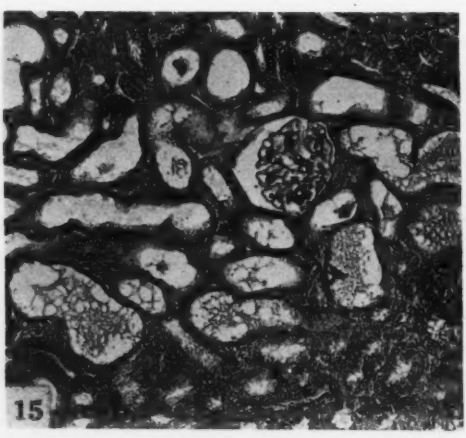

FIG. 15. Another kidney 5 minutes after alloxan showing general distension and a flocculent material in more distal elements of the nephron, lower left. Aldehyde fuchsin. ×420.

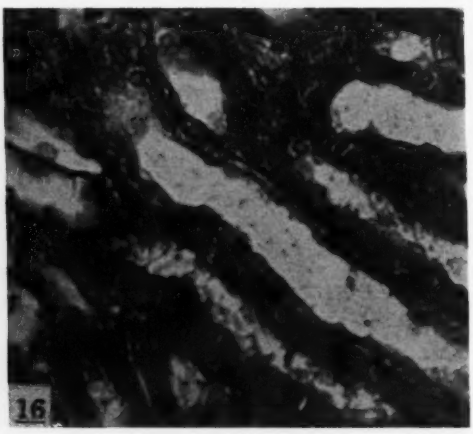

FIG. 16. Section through a medullary ray of still another kidney 5 minutes post alloxan showing distension of Henle's loop. Aldehyde fuchsin. ×420.

Both observations suggest increased secretory activity. Further study of mitochondria and Golgi substance may clarify this question.

There is more than one explanation for areolation. It is tempting to accept this as evidence for release of secretion, and consider the appearance of exaggerated pericapillary (15) and interceullular (6, 7) spaces due to accumulation of products discharged from beta-cells. The mere fact that blood sugar continues its ascent need not preclude this suggestion, since glycogenolysis may be even more rapid, with consequent depletion of liver glycogen. Although the fact that practically every beta-cell retains its high granule concentration as areolation proceeds seems to militate against release, our GCA material shows the same constant pattern of hyperactivity observed earlier, an indication that the rate of synthesis may equal that of discharge. It must be considered, however, that the areolated appearance may be due to edema (15).

Our studies show that, during the early degeneration phase, we are dealing for the first time with markedly abnormal cells, whereas most previous investigators, whose classical descriptions of events in other animals are well-known, place the inception of degenerative lesions within the first hour (1, 6, 15). Actually, our GCA series indicates that degeneration is preceded by a period of reduced insulin synthesis, and even this does not occur until at least 2 hours have elapsed. Active degeneration appears several hours later.

In most of the earlier accounts, the hypoglycemic phase is explained by the release of insulin from disintegrating cells. Apparently, this plays a part in the persistence of low sugar levels. We suggest, however, that this entire phenomenon becomes much more comprehensible when it is considered that oversecretion and exhaustion of liver glycogen occur before the onset of degeneration.

Although the substitution process has been described, the source of these mononuclear cells is not mentioned. Their origin from perivascular and periinsular connective tissue is not surprising. Their final disposition awaits further study.

The histological picture of the liver in the alloxan diabetic animal has often been presented. A description of changes during the first hours is, however, rare. The same may be said of the estimation of glycogen. In most reports, chemical analysis, rather than histochemical determination,[7] was used and few investigations correlate glycogen content with the blood sugar curve. In 1944, Duff and Starr stated that the liver is histologically lacking in glycogen, but the time after treatment was indefinite. Herbut *et al.* (14) described a coagulative degeneration and necrosis in the rabbit 5 to 10 hours after alloxan and add that this is associated with lack of

[7] Bondy *et al.* (4) among others showed a close correlation between the quantitative determination of glycogen, chemically, and the histochemical preparations estimated by optical density.

glycogen. During this same period, many of our animals showed low glycogen, but no degeneration was seen. Pascual-Lopez (20), using chemical analysis, reported a loss of liver glycogen and found the maximum decrease 90 minutes after alloxan. This coincides with our findings.

The only reference to nucleic acids in the liver (3) mentioned an increase in DNA. Although DNA is not included in this report, our observations show marked similarity between glycogen and RNA (liver basophilia Fig. 1) curves, particularly during glycogenolysis. Conversely, the reappearance of RNA precedes glycogenesis.

Since 1943 when Dunn and McLetchie reported kidney lesions, descriptions ranging from slight to severe tubular degeneration have appeared. Among these accounts, Herbut *et al.* (14) mention renal changes during the first day. Our findings agree with theirs only in the appearance of the granular substance in the lumena of certain tubules, degenerative changes being absent at this time in our animals. Such transformations as were found could be explained by a marked rise in blood and filtration pressure, which tends to balloon capillaries, capsule and tubules alike.

Goldner and Gomori (12) observed glycogen in the kidneys of diabetic dogs. Later, Curtis *et al.* (5) and Robbins (21) were able to correlate this glycogen nephrosis to a critical level in hyperglycemia, reported to be 350 mg.%. However, their work was based upon animals in which diabetes had been well-established for some time.

SUMMARY

This report indicates that: during initial hyperglycemia, liver glycogen is almost completely lost and nucleic acids are greatly reduced; beta-cells lose their ability to synthesize insulin between 2 and 5 hours; between 5 and 24 hours, 95% of the beta-cells have degenerated and been replaced by mononuclear cells; after 9 hours, hepatic nucleic acids approach normal, but, like glycogen, show much variability for many hours; kidney changes are characteristic of hyperactivity and high pressure but are not degenerative. It is suggested that evidence of an initial beta cell stimulatory effect of alloxan is found in the early increase in beta-cell granulation, accompanied by vascular engorgement and increased cytoplasmic nucleic acid.

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THE EFFECT OF GROWTH HORMONE ON HEMATOPOIESIS IN HYPOPHYSECTOMIZED ADULT FEMALE RATS[1]

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THE fact that hypophysectomy is followed by an anemia is well established. This has been found to be true for every animal investigated as well as for the human being. Growth hormone has been one of several hormones used as a therapy to prevent this anemia but with confusing results. Meyer, Stewart, Thewlis, and Rusch (1) found that growth hormone would stimulate bone marrow so that the reticulocyte number was greatly increased, but the erythrocyte count was not altered. Gaebler and Mathies (2) found that growth hormone had no effect on the hemoglobin level of hypophysectomized rats. Van Dyke, Simpson, Garcia, and Evans (3) also were unable to prevent post-hypophysectomy anemia with daily injections of 1.25 mg. of growth hormone for 30 days. The erythrocyte count, hematocrit percentage, hemoglobin level, and the total cell volume per 100 gm. of body weight were not changed from levels exhibited by the the untreated hypophysectomized rats. Crafts (4) reported similar results with gradually increasing doses of growth hormone, starting with 0.1 mg. per day and gradually increasing to 0.8 mg. daily. Gemzell and Sjöstrand (5) measured the total amount of hemoglobin in hypophysectomized rats and in hypophysectomized rats treated with growth hormone. The total hemoglobin was increased in the treated rats, but only to a degree that corresponded with the increase in body weight of the animals. Fruhman, Gerstner, and Gordon (6) have found growth hormone to be similarly ineffective insofar as repair of the peripheral blood picture is concerned, but claim that this ineffectiveness is only apparent because a hemodilution accompanies growth hormone therapy and is responsible for the persistence of the anemia. As support for their contention that growth hormone stimulates erythropoiesis in the hypophysectomized rat, they present an elevated reticulocyte count and a hyperplastic bone marrow with an increase in erythroid elements. In contrast to two of the above reports (1, 6), Overbeek (7), and Querido and Overbeek (8) found that growth hormone had no influence on the reticulocyte count of hypophysectomized rats.

The present investigation was undertaken in an attempt to resolve this

Received May 11, 1956.

[1] This investigation was supported by a research grant (A476) from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service.

[2] With the technical assistance of Mrs. Barbara Giffen and Robert Fischoff. The photography was done by Mr. Joseph Homan of the Medical Art Department.

discrepancy in the literature, and to add further details concerning the effect of growth hormone on hematopoiesis.

MATERIAL AND METHODS

Adult female rats of 3 to 4 months-of-age and of the Sprague-Dawley strain were used in this experiment. All rats were fed standard Purina chow ad libitum supplemented once a week with lettuce. The completeness of hypophysectomies was checked by examination of the organ site at autopsy with a binocular dissecting microscope. Blood was obtained by anesthetizing the rat with ether, clipping the tail, and collecting 5 or 6 drops in a small vial containing a minute amount of heparin. Erythrocyte and white cell counts were done in duplicate, using U. S. certified blood pipettes and the improved Neubauer counting chamber. Hemoglobin determinations were made in a Klett-Summerson photoelectric colorimeter. Hematocrit determinations were made with Van Allen hematocrit tubes (no diluent) and spun for 1 hour at 2000 r.p.m. Blood volume determinations were made by the Evans Blue dye method as described by Metcoff and Favour (9).

Bone marrow was obtained from both femurs and utilized as follows: The right femur was used to determine the total number of nucleated marrow cells and for differential counts. The rat was etherized, bled from the tail, killed with chloroform, and the right femur removed. The blood removed was centrifuged to provide homologous serum for a diluent. The femur was split and the marrow mass placed on a glass slide. Exactly 10 cu. mm. of marrow were drawn into a calibrated, siloconized pipette, and then expelled into a small vial and diluted with 0.5 cc. of the homologous serum. Any adhering tissue in the pipette was washed out by drawing the serum up and down into the pipette. An even suspension was obtained by the gentle aspiration and expulsion of the mixture with a rubber-nippled pipette. A sample of the suspension was used to make smears for the differential counts, these smears being stained later with Giemsa buffered to pH 5.3. From the remainder, 4 samples were drawn into white blood cell pipettes, diluted with Randolph's staining fluid, and hemocytometer counts made of the nucleated cells. The average was then multiplied by 50 to make up for the diluent added to the original bone marrow. The resultant figure was the total number of nucleated cells per cu. mm. of bone marrow tissue. The left femur was removed, the ends cut off and one side of the shaft cut away. This was placed in Helly-fixative, later washed and put in 70% alcohol. Subsequently the marrow was removed from the bone in a long strip, cleared in Dioxane, embedded in paraffin, sectioned at 6 micra, mounted and stained with Giesma stain.

The rats have been divided into 3 groups. The first received no treatment and served as normal controls, the second was hypophysectomized and autopsied 125 days after surgery, and the third group was hypophysectomized, left unmolested for 75 days, and then treated with growth hormone for 50 days. Blood was examined at 10-day intervals during treatment. Growth hormone[3] was given daily in gradually increasing amounts as follows: days 1–9, 0.1 mg.; days 10–19, 0.2 mg.; days 20–29, 0.3 mg.; days 30–39, 0.4 mg.; and days 40–49, 0.8 mg.

RESULTS

The results on body weight are presented in Table 1; the erythrocyte picture, Table 2; total plasma and cellular volumes, Table 3; total white cell count, Table 4; total nucleated cells in the bone marrow, Table 5;

[3] We wish to thank Armour and Co. for the growth hormone (Armour lyophilized growth hormone Lot No. R377237) used in this experiment.

TABLE 1. BODY WEIGHTS OF 3 GROUPS OF ADULT FEMALE RATS: (1) NORMAL CONTROLS, (2) HYPOPHYSECTOMIZED FOR 125 DAYS, AND (3) HYPOPHYSECTOMIZED FOR 75 DAYS FOLLOWED BY GROWTH HORMONE TREATMENT FOR 50 DAYS. ± = STANDARD ERROR

	Normal controls	Hyp., no treatment		Hyp., growth hormone	
	28 rats	22 rats	% of normal	15 rats	% of normal
Body Weight in grams	286.3 ±4.6	215.6 ±2.8	75	268.7 ±4.0	94

TABLE 2. THE ERYTHROCYTE PICTURE IN 3 GROUPS OF ADULT FEMALE RATS: (1) NORMAL CONTROLS, (2) HYPOPHYSECTOMIZED FOR 125 DAYS, AND (3) HYPOPHYSECTOMIZED FOR 75 DAYS FOLLOWED BY GROWTH HORMONE TREATMENT FOR 50 DAYS. ± = STANDARD ERROR

	Normal controls	Hyp., no treatment		Hyp., growth hormone	
	28 rats	22 rats	% of normal	15 rats	% of normal
Reticulocytes in percent	0.3 ±0.08	0.3 ±0.06	100	2.1 ±0.3	600
Erythrocytes in millions per cu. mm.	8.05 ±0.09	6.90 ±0.16	86	6.67 ±0.12	83
Hematocrit in percent	44.8 ±0.3	38.6 ±0.4	86	37.1 ±0.5	83
Hemoglobin in gm. per 100 cc.	15.6 ±0.11	13.4 ±0.12	86	12.4 ±0.2	79
Mean Corpuscular Volume in cu. microns	55.6	55.9	101	55.6	100
Mean Corpuscular Hemoglobin in micromicrograms	19.3	19.4	101	18.5	96
Mean Corpuscular Hemoglobin Concentration in Percent	34.8	34.7	99	33.4	96

TABLE 3. BLOOD AND PLASMA VOLUMES OF 3 GROUPS OF ADULT FEMALE RATS: (1) NORMAL CONTROLS, (2) HYPOPHYSECTOMIZED FOR 125 DAYS, AND (3) HYPOPHYSECTOMIZED FOR 75 DAYS FOLLOWED BY GROWTH HORMONE TREATMENT FOR 50 DAYS. ± = STANDARD ERROR

	Normal controls	Hyp., no treatment		Hyp., growth hormone	
	24 rats	19 rats	% of normal	14 rats	% of normal
Plasma Volume in cc.	8.44 ±0.17	6.23 ±0.12	74	7.90 ±0.12	94
Plasma Volume in cc. per 100 gm. body weight	3.01 ±0.067	2.95 ±0.07	98	2.96 ±0.20	98
Total Cellular Volume in cc.	6.35 ±0.16	3.55 ±0.09	56	3.78 ±0.19	60
Total Cellular Volume in cc. per 100 gm. body weight	2.27 ±0.06	1.68 ±0.04	74	1.41 ±0.06	62

TABLE 4. TOTAL WHITE CELL COUNTS OF 3 GROUPS OF ADULT FEMALE RATS: (1) NORMAL CONTROLS, (2) HYPOPHYSECTOMIZED FOR 125 DAYS, and (3) HYPOPHYSECTOMIZED FOR 75 DAYS FOLLOWED BY GROWTH HORMONE TREATMENT FOR 50 DAYS. ± = STANDARD ERROR

	Normal controls	Hyp., no treatment		Hyp., growth hormone	
	25 rats	22 rats	% of normal	14 rats	% of normal
Total White Cell Count in thousands per cu. mm.	12.4 ±0.7	15.4 ±0.5	124	16.9 ±1.0	136

TABLE 5. THE TOTAL NUMBER OF NUCLEATED CELLS IN THE BONE MARROW OF 3 GROUPS OF ADULT FEMALE RATS: (1) NORMAL CONTROLS, (2) HYPOPHYSECTOMIZED FOR 125 DAYS, AND (3) HYPOPHYSECTOMIZED FOR 75 DAYS FOLLOWED BY GROWTH HORMONE TREATMENT FOR 50 DAYS. ± = STANDARD ERROR

	Normal controls	Hyp., no treatment		Hyp., growth hormone	
	25 rats	19 rats	% of normal	14 rats	% of normal
Total Nucleated Cells in millions per cu. mm. of marrow tissue	1.774 ±0.045	1.309 ±0.045	74	2.268 ±0.060	128

differential counts of marrow cells, Table 6; and the number of each cell type in the bone marrow per cu. mm. of marrow tissue, Table 7.

Body Weight

Hypophysectomy induced the expected decrease in weight of the animals to 75% of normal (Table 1). Growth hormone treatment returned the body weight to an average which was 94% of normal. The growth hormone therapy, therefore, was sufficient to elevate the body weight to normal levels.

The Erythrocyte Picture

The data in Table 2 show that hypophysectomy induced the usual anemia. The 14% decrease in erythrocyte count is similar to results already reported with this strain of rats (10), but the similar decreases in hematocrit and hemoglobin levels are not as great as found previously. In addition, the data show that the anemia was of the normocytic-normochromic type in contrast to our previous report (10) which indicated a slightly microcytic-hypochromic type of an anemia.

Growth hormone treatment induced an increase in reticulocyte count. In spite of this increase, the erythrocyte count, hematocrit, and hemoglobin levels remained at values resembling the hypophysectomized rats with no treatment.

Blood and Plasma Volumes

The data in Table 3 show that hypophysectomy induced a decrease in plasma volume and that growth hormone restored plasma volume to nor-

TABLE 6. DIFFERENTIAL BONE MARROW COUNTS OF 3 GROUPS OF ADULT FEMALE RATS: (1) NORMAL CONTROLS, (2) HYPOPHYSECTOMIZED FOR 125 DAYS, AND (3) HYPOPHYSECTOMIZED FOR 75 DAYS FOLLOWED BY GROWTH HORMONE TREATMENT FOR 50 DAYS. ALL FIGURES IN PER CENT. ± = STANDARD ERROR

	Normal controls	Hyp., no treatment		Hyp., growth hormone	
	21 rats	20 rats	% of normal	13 rats	% of normal
Stem Cells	3.6 ±0.07	3.6 ±0.22	100	3.2 ±0.4	89
Neutrophilic Elements	30.7 ±1.33	33.2 ±0.63	108	26.7 ±1.6	87
Eosinophilic Elements	5.8 ±0.31	3.9 ±0.23	67	3.6 ±0.2	62
Proerythroblasts	1.8 ±0.13	2.5 ±0.13	139	2.1 ±0.16	117
Erythroblasts	26.0 ±1.23	26.3 ±0.91	101	28.5 ±1.0	110
Normoblasts	23.3 ±1.51	22.5 ±1.52	97	29.2 ±1.7	125
Lymphocytes	2.3 ±0.17	3.9 ±0.42	170	1.9 ±0.18	83
Other Cells	0.9 ±0.12	0.6 ±0.08	66	0.6 ±0.12	66
Questionable Cells	4.6 ±0.33	4.5 ±0.17	98	4.0 ±0.64	87
Total Erythroid Elements	51.2 ±1.55	51.2 ±1.61	100	59.8 ±2.0	117
Total Myeloid Elements	37.0 ±1.37	36.1 ±1.45	98	30.3 ±1.6	82

mal values. It should be noted, however, that these changes correspond to the decrease and increase in body weight which occurred following hypophysectomy and subsequent treatment with growth hormone. When body weight was taken into consideration, plasma volumes remained normal. The total cellular volume, on the other hand, decreased to 56% of normal after hypophysectomy, and remained below normal (74% of normal) when the weight of the rats was taken into consideration. Growth hormone therapy did not change the total cellular volume, for it remained low (60% of normal) and was equally low (62% of normal) when cellular volume was calculated per 100 gm. of body weight.

Total White Cell Count

Hypophysectomy was followed by a moderate increase (24%) in white cell count (Table 4) and this increase was maintained in the rats receiving growth hormone therapy, the latter exhibiting averages 36% above normal.

TABLE 7. THE NUMBER OF MARROW CELLS PER CU. MM. OF MARROW TISSUE OF 3 GROUPS OF ADULT FEMALE RATS: (1) NORMAL CONTROLS, (2) HYPOPHYSECTOMIZED FOR 125 DAYS, AND (3) HYPOPHYSECTOMIZED FOR 75 DAYS FOLLOWED BY GROWTH HORMONE TREATMENT FOR 50 DAYS. ALL FIGURES EXPRESSED IN TERMS OF THOUSANDS OF CELLS PER CU. MM

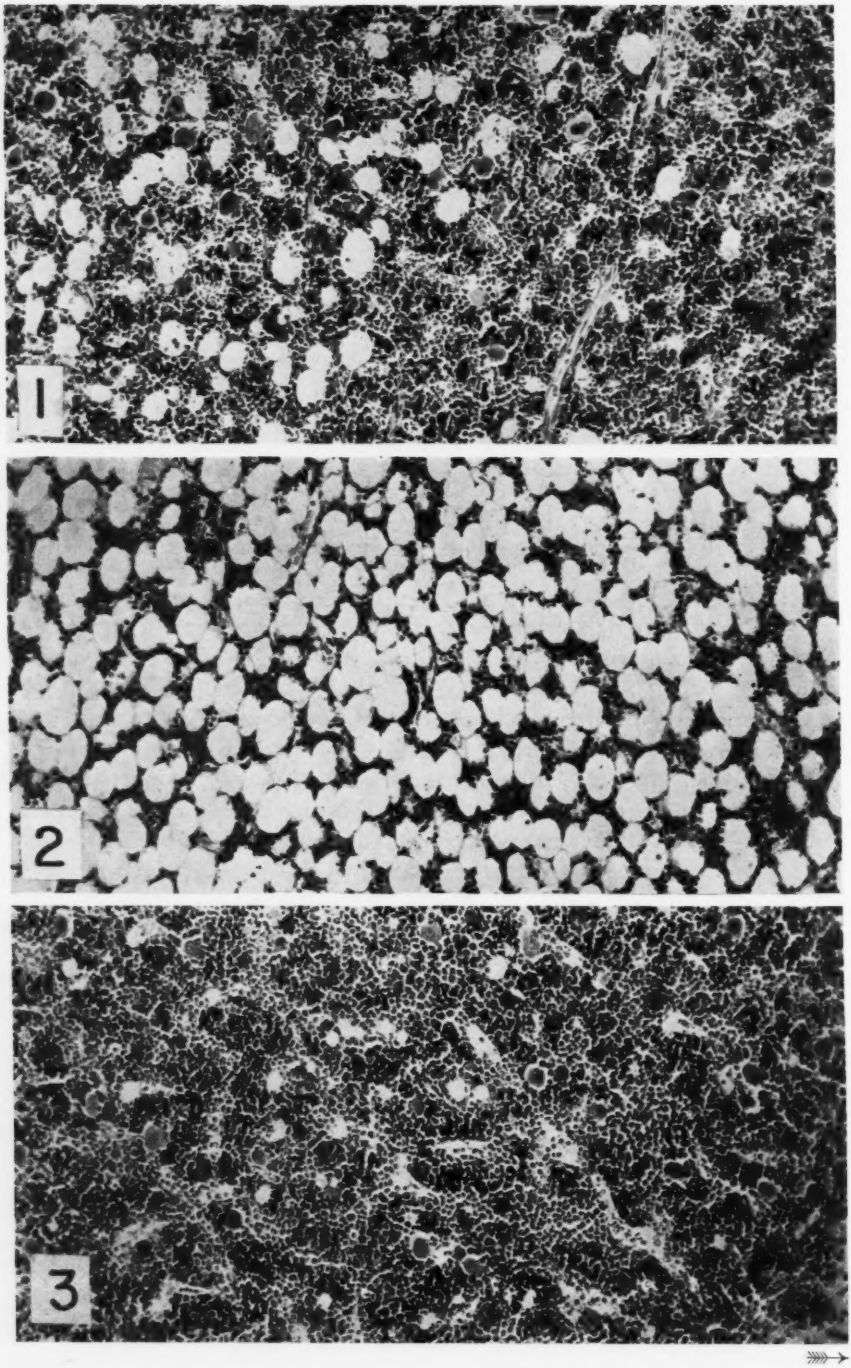
	Normal controls	Hyp., no treatment		Hyp., growth hormone	
	21 rats	20 rats	% of normal	13 rats	% of normal
Stem Cells	63.9	47.1	74	72.6	114
Neutrophilic Elements	544.7	434.6	80	605.6	111
Eosinophilic Elements	102.9	51.1	50	81.6	79
Proerythroblasts	32.0	32.7	102	47.6	149
Erythroblasts	461.2	344.3	75	646.4	140
Normoblasts	413.3	294.5	71	662.3	160
Lymphocytes	40.8	51.1	125	43.1	106
Other Cells	16.0	7.9	49	13.6	85
Questionable Cells	81.6	58.9	72	90.7	111
Total Erythroid Elements	908.3	670.2	74	1356.3	149
Total Myeloid Elements	656.4	472.6	72	687.2	105

Bone Marrows

Observations on microscopic sections of the bone marrow (Figs. 1, 2, and 3) showed that the normal rat has a marrow in which the islands of erythroid elements are easily seen, and which contains a moderate amount of fat. Hypophysectomy induced a decrease in size and clarity of the erythroid islands, and an increase in fat. Growth hormone had a profound effect on the bone marrow; most of these marrows were called hyperplastic or normal upon examination. The islands of erythroid tissue were enlarged and the amount of fat was decreased; the marrows were hyperplastic.

Table 5 gives the number of nucleated cells per cubic millimeter of marrow tissue and these data support the histologic picture just described. Hypophysectomy induced a 26% decrease in nucleated cells per cu. mm. of marrow tissue and growth hormone not only restored the marrow cell number to normal but caused a 28% increase when compared to the normal animals, and a 54% increase over the number of marrow cells in the non-treated hypophysectomized rats.

Table 6 presents data obtained from differential counts of the bone marrows, and, contrary to a previous report (10) and in agreement with Landau and Gordon (11), hypophysectomy did not induce decreases in the percentage of erythroid elements; the percentages of all stages in the erythroid series and total myeloid elements were normal. Growth hormone induced increases in the percentage of proerythroblasts, erythroblasts and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normoblasts, and, therefore, in total erythroid elements; the myeloid elements exhibited a relative decrease in number. Although it is difficult to categorize the various developmental stages of the erythroid series in the rat, the results indicated that the entire sequence from immature to mature forms was represented under each experimental condition.

As the number of cells per cu. mm. of marrow (Table 5) and the percentage of each cell type (Type 6) were determined, the number of each cell type studied per cu. mm. of marrow tissue was obtained by simply multiplying the numbers in Table 6 by those in Table 5. These data are presented in Table 7 and show that hypophysectomy actually caused decreases in all cell types except the proerythoblasts and the lymphocytes; the total erythroid elements and total myeloid elements were decreased to levels 74 and 72% of normal respectively. Growth hormone increased the number of cells in the bone marrow, the erythroid elements responding to a greater degree than the myeloid elements; there was a 49% increase in the former and a 5% increase in the white cell precursors. When compared to the hypophysectomized animals with no treatment, these increases are 75% and 33% respectively.

In summary, hypophysectomy induced a decrease in the number of erythroid elements in the bone marrow which was reflected in peripheral decreases in erythrocyte count, hematocrit, and hemoglobin levels; the anemia was of the normocytic-normochromic type. Growth hormone therapy in hypophysectomized rats induced marked increases in the number of erythroid elements in the bone marrow. Although reticulocyte counts were elevated, this therapy did not eliminate the anemia in these animals. Blood volume studies indicated that this lack of an effect on the peripheral blood could not be accounted for by a hemodilution; the plasma volume increased but only in ratio to the increase in body weight of the injected rats.

DISCUSSION

These data have shown that hypophysectomy induces an anemia and that growth hormone will not eradicate it in spite of the fact that the bone marrow is stimulated to such an extent that there is a 28% increase in the number of cells per cubic millimeter of marrow tissue when compared to the normal rats, and a 54% increase when compared to the hypophysectomized animals which received no treatment. In addition, the stimulation of the erythroid elements was greater than that observed in the myeloid series of cells, and the reticulocyte percentage was increased.

FIG. 1. Bone marrow from a normal control rat. ×160

FIG. 2. Bone marrow from a rat hypophysectomized for 125 days. ×160

FIG. 3. Bone marrow from a rat hypophysectomized for 75 days followed by growth hormone treatment for 50 days. ×160.

An explanation for a stimulated bone marrow and an increase in reticulocytes not being accompanied by an increase in peripheral blood erythrocytes is difficult. Fruhman, Gerstner, and Gordon (6) explain this by claiming a hemodilution accompanies growth hormone therapy. The present investigation does not support this view. There is a possibility that the bone marrow is delivering erythrocytes at a rate equal to the increased need for them which the increase in weight of the animal demands. This would agree with the work of Gemzell and Sjöstrand (5) who found that growth hormone injections increased hemoglobin in hypophysectomized rats to a degree equal to body weight increases. The lack of an increase in total cellular volume found in the present investigation does not indicate that any great number of cells were delivered to the peripheral blood.

The role of thyroxin is of interest. Meyer *et al.* (1) found that growth hormone alone would not alter the peripheral erythrocyte number in spite of a reticulocytosis; if thyroxin was added to the somatotropin, however, a response in peripheral blood was obtained. Meineke and Crafts (12) have found a similar phenomenon in hypophysectomized rats treated with cortisone. Although the bone marrow in the cortisone-treated hypophysectomized rats was entirely different in appearance than exhibited by those treated with growth hormone, a similar failure to eliminate the anemia occurred. When thyroxin was combined with the cortisone, the anemia was eliminated. This beneficial role of thyroxin is being investigated.

SUMMARY

Adult female rats of the Sprague-Dawley strain were divided into 3 groups: (1) normal controls, (2) hypophysectomized for 125 days, and (3) hypophysectomized for 75 days followed by growth hormone therapy for 50 more days. Hypophysectomy induced a hypoplasia of the bone marrow which was due to a 26% decrease in total nucleated cells per cubic millimeter of marrow tissue; this decrease consisted of a loss in both erythroid and myeloid elements. These changes were accompanied by a decrease in peripheral erythrocytes, hematocrit reading, and hemoglobin values of 14%; the anemia was of the normocytic-normochromic type.

Growth hormone therapy in hypophysectomized anemic rats produced a hyperplasia of the bone marrow, the erythroid elements responding in a more marked fashion than the myeloid elements; the changes were accompanied by an increase in reticulocyte percentage. In spite of this growth of the marrow, the peripheral anemia was not altered; blood volume studies did not support the view that this lack of effect on the peripheral blood picture was due to a hemodilution.

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AN ACCESSORY ADRENAL CORTEX IN THE KIDNEY OF A RHESUS MONKEY AND THE EFFECTS OF PYRIMETHAMINE ON THIS TISSUE

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THE frequent occurrence of accessory adrenal tissue is well known, since a statement or summary of its constituents and locations appears in most textbooks dealing with adrenal glands. In brief review, it may be stated that the accessory bodies may be made up of either cortical tissue or medullary tissue alone, or a combination of both, the latter sometimes resembling true adrenal glands in miniature. Such accessory bodies may be located in the capsule of the adrenal glands themselves, in the fat and connective tissue around the adrenals and cranial portions of the kidney, occasionally within the kidney, in the retroperitoneal area below the kidneys, in the broad ligament, in the scrotum, and sometimes in other regions of the abdominal cavity. It is assumed that some of these may be functional; in some animals, at least, indirect evidence for this is strong.

In recent years we have studied sections of several hundred kidneys and adrenals (and other organs) of rhesus monkeys following various experimental procedures. No accessory adrenal tissue has been encountered, until the one to be reported, except for the fairly common occurrence of islands or cords of cortical tissue in the capsule of the adrenal glands proper. This report deals with a miniature accessory adrenal cortex, found in the peripheral cortex of a kidney of a rhesus monkey, completely enclosed within the kidney capsule, and the effects of the antimalarial drug pyrimethamine on this tissue.

EXPERIMENTAL PROCEDURE

Monkey 6383 was one of a group which had been treated with daily doses of 5.0 mg. per kg. body wt. of pyrimethamine (2,4-Diamino-5-p-chlorophenyl-6-ethylpyrimidine, formerly known as Daraprim),[1] an antimalarial drug with considerable toxicity. With this dosage, it induces changes in a number of organs and usually causes death (1). This animal had shown characteristic pyrimethamine reactions and died on the 36th day of treatment.

Our usual procedure, in preparing slides of organs for microscopic study, is to section

Received May 25, 1956.

[1] This monkey was one of a group examined as part of a cooperative study of the toxicity of pyrimethamine carried out at the Christ Hospital Institute of Medical Research, Cincinnati, and in the Department of Anatomy, University of Cincinnati College of Medicine.

well into the block of tissue, continue cutting for a time mounting every 20th section and discarding the ones in between, and then to save the remaining block of tissue. Part of this adrenal happened to be in the first portion of the kidney block, so that every 20th section only was available. However, serial sections were made of the remaining tissue. The majority was stained with Lillie's hematoxylin and eosin, a few with Maximow's stain.

GENERAL DESCRIPTION OF THE ACCESSORY ADRENAL

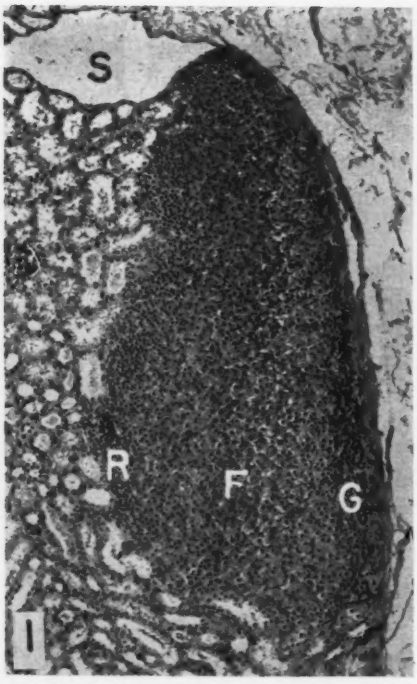
Figure 1 shows the accessory adrenal at its maximum diameters, 1 mm. $\times$0.5 mm. It was located at one end of the kidney, presumably the apical pole. The capsule of the kidney also formed the capsule for the accessory adrenal. At its upper margin, the adrenal tissue was in immediate contact with a stellate vein. Elsewhere the adrenal was immediately adjacent to kidney tissue, to tubules of either cortical labyrinths or medullary rays (Fig. 2). Sometimes a few reticular fibers, or a capillary, were seen between the kidney and adrenal tissues. Only one adjacent renal corpuscle was seen, the parietal layer of Bowman's capsule being in contact with fascicular cells in a small area where the reticular zone appeared to be absent. One proximal and three distal convoluted tubules were found in the midst of the adrenal tissue, all being extensions of tubules in the adjacent kidney cortex.

In one region of the accessory adrenal, the glomerular zone was absent in part, but then appeared as a narrow zone which graded into a progressively wider area (Figs. 1 and 2). Elsewhere in the gland the glomerular zone was continuous, although it was reduced to a thin strand of cells in one area. Near this area, in the capsule, were two separate cords of glomerular cells, both of which became continuous later with the glomerulosa. For the most part, the glomerular cells were arranged in typical ovoid groups or in occasional cords. The cells of the fascicular zone showed the characteristic arrangement into cords. The cells decreased rapidly in size toward the bottom of this zone, and merged with the small-celled reticular zone, which was present throughout the greater part of the gland.

Between the various cords of cells were capillaries or sinuses, lined in part with reticuloendothelial cells, as in typical adrenal glands. Continuity of such vessels with capillaries or small venules of the kidney cortex or of the capsule could be seen in several places. One such capillary, lying between a distal convoluted tubule and fascicular cells, opened into the stellate vein. Other vessels were not seen. The accessory adrenal, therefore, appeared to be supplied by extensions of the cortical capillaries of the kidney.

HISTOLOGICAL STRUCTURE OF THE ADRENAL GLANDS PROPER AND THE ACCESSORY ADRENAL

The adrenal glands proper showed a typical response to the toxicity of pyrimethamine (Fig. 3). As compared with adrenals of untreated control animals (Fig. 4), the glomerular zone was decreased in width and showed an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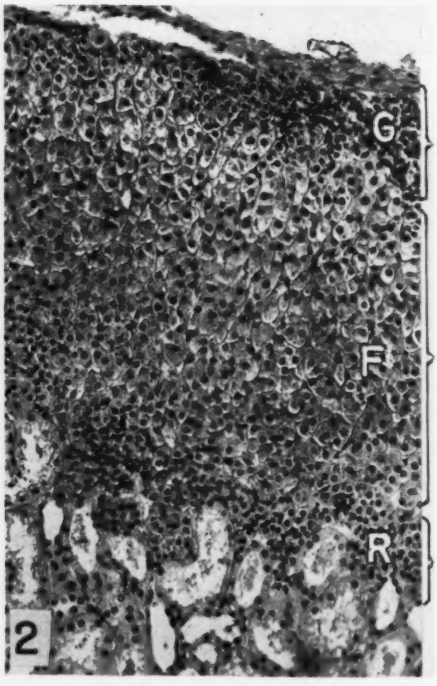
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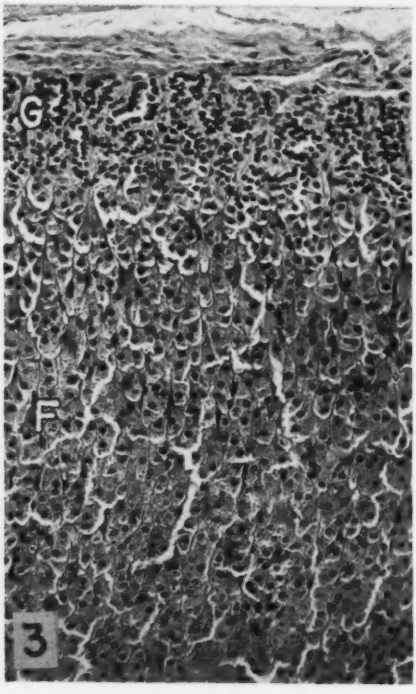
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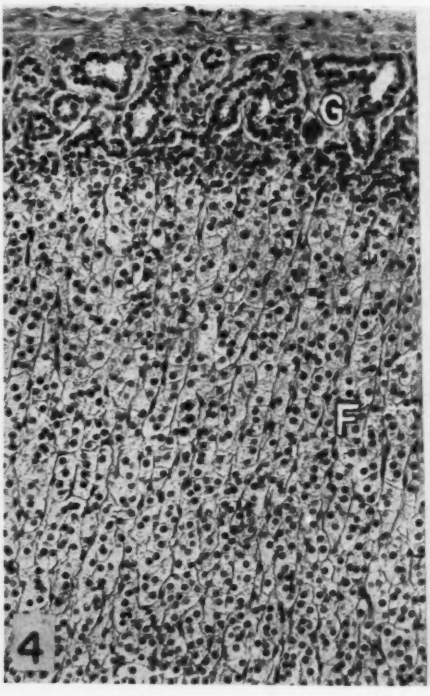
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uneven, indistinct junction with the fascicular zone. Glomerular cells were smaller due to loss of the normal fine lipid vacuolation; nuclei were smaller due to condensation of chromatin. Cell cords were usually compact. The fascicular zone, however, was increased in size, thereby causing an increase in size and weight of the entire gland. These cells were somewhat enlarged, and had compactly granular cytoplasm which showed none of the characteristic vacuolation seen in control adrenals. Nuclei were more variable in size and form, many being less vesicular than in controls. There were no specific alterations in the reticular zone.

The histological structure of the accessory adrenal cortex embedded in the kidney was similar to that of the adrenal glands proper (compare Figs. 1 and 2 with Fig. 3) and not like that of the glands of normal untreated monkeys (Fig. 4). The absence of a glomerular zone in places and marked irregularity in width of this zone, as seen in this accessory adrenal, are common findings in the adrenals of pyrimethamine-treated monkeys. Both features happened to be less pronounced than usual in the adrenals proper of this animal. The slight lipid vacuolation of fascicular cells in the transitional zone of the accessory gland was also seen in some areas of the adrenals proper, although not in the section illustrated in Fig. 3. Fascicular cells of the accessory gland and the adrenals proper were similar in size and structure. Reticular cells of the accessory gland were somewhat smaller.

SUMMARY

Accessory adrenal tissue, embedded within the peripheral cortex of the kidney of a rhesus monkey, exhibited glomerular, fascicular and reticular zones as in adrenal glands proper. The monkey had been treated with the antimalarial drug pyrimethamine. This drug evoked changes in the accessory adrenal tissue which were similar to those found in the cortex of the adrenal glands proper. The accessory tissue, therefore, responded in the same way as did the adrenal glands.

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All sections paraffin stained with hematoxylin and eosin. S = stellate vein; G = glomerular zone; F = fascicular zone; R = reticular zone.

Fig. 1. Accessory adrenal cortex in the kidney, monkey 6383, after pyrimethamine treatment, ×80.

Fig. 2. Accessory adrenal cortex in the kidney, monkey 6383, after pyrimethamine treatment, ×160.

Fig. 3. Adrenal gland proper, monkey 6383, after pyrimethamine treatment, ×160.

Fig. 4. Adrenal gland proper, monkey 6098, untreated control, ×160.

IN VITRO EFFECTS OF STEROIDS UPON ELECTROLYTE TRANSFER THROUGH FROG SKIN

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THE effects of adrenal cortical steroids upon water and electrolyte shifts in the organism have been mainly ascribed to their action upon the kidney. Extensive clinical and experimental findings have pointed to the role of the glomeruli and the tubular apparatus in retaining sodium and water and excreting potassium (1). However, as early as 1936 Swingle and co-workers (2, 3) deduced that some of the changes must take place in extrarenal tissues, a hypothesis which was confirmed later (4). A direct approach to this problem was attempted by Woodbury and associates who studied salt absorption from the intestinal tract (5), and shifts of electrolytes in skeletal muscles of nephrectomized animals (6).

The surviving frog skin preparation seemed to be potentially suitable for the study of the action of steroids upon electrolyte transfer by an isolated system. The properties of such preparations have been extensively reviewed by Huf (7), and the kinetics and mode of exchanges have been examined by Ussing (8), Linderholm (9) and others (7). Surviving frog skin under favorable conditions may for many hours take up sodium from the surrounding Ringer's solution. It was of interest to learn whether addition of steroid hormones *in vitro* would alter the rate of electrolyte transfer. The data to be presented in this report definitely indicate that various steroids can directly affect the transfer system of the isolated frog skin.

METHODS

The methods employed in our studies were essentially those described by Huf and co-workers (10). Male frogs (Rana pipiens) were maintained in cool surroundings and received no food. On the day of the experiment, the frogs were pithed, and the skin was stripped from the hind legs in such a fashion that both the right and left leg skins could be made into bags by appropriate trimming and ties. In all cases, the epithelial surface formed the outside surface of the bag. A wide, short cannula was fastened into the upper aperture of the bag and the whole preparation was suspended in slightly hypotonic Ringer's solution. Four ml. of the same solution was pipetted into the bag, which was adjusted so that the inner and the outer fluid levels were equal. Oxygen was bubbled through the outside container throughout each experiment.

Received May 31, 1956.

[1] The department is in part supported by the Medical Research Institute, Michael Reese Hospital, Chicago, Illinois. The work was aided by a grant (A-30) from the U. S. Public Health Service.

Frog Ringer's solution containing approximately 196–200 milliosmols per liter was used in all experiments. The incubation time was 16 hours. At the beginning and the end of all experiments, the number of milliosmols in samples of the inside and outside solutions were calculated by determining the freezing point depression of the various fluid specimens with the aid of a Fiske Osmometer. The skin surface of each bag was estimated by weighing out tracings of the skin on standard graph paper. All values were then calculated and recorded as net milliosmols transferred per cm^2 of the skin. In every case, the control skin bag was compared with the opposite experimental skin bag taken from the same frog. As would be expected from previous reports (7), frog skin bags varied in their concentrating capacity widely from animal to animal. The experimental periods involved in the present study ran from November to April, and the seasonal changes may have influenced individual performances. The results have been consequently recorded as differences in milliosmols transferred per square centimeter between the experimental control skin bags of each set of preparations from a single frog. It was hoped that the wide range of absolute values existing between animals would thereby be obviated, and each animal would serve as its own control.

The experimental frog skin bags contained the following materials: plain Ringer's, plain Ringer's + solvent alone; Ringer's + solvent containing steroids. The steroids tested included 9-α-fluorohydrocortisone, free desoxycorticosterone, hydrocortisone, estriol and testosterone. The concentrations of these substances were either 0.2, 2.0, or 20 μg. per ml. of Ringer's solution inside the bag, with the total volume being held constant at four ml. Crystalline desoxycorticosterone, estriol and testosterone were dissolved in a solvent, which consisted of propylene glycol and benzyl alcohol with Tween 80 added.[2] The hydrocortisone and fluorohydrocortisone were supplied commercially in solution. An attempt was made to determine the fluid volume in the interior of the frog skin bags at the end of the experiment by weighing the total fluid contents.

RESULTS

In all instances the control skin bags containing the Ringer's solution alone concentrated electrolytes in the interior, as was demonstrated by a definite increase in the interior osmotic pressure. A considerable variation of the individual animals was evident, as observed by previous investigators. The lowest control value for the difference of the inside vs. the outside fluid was 0.6 milliosmols, while the highest was 1.7 milliosmols per square centimeter.

Table I demonstrates the difference between the untreated and treated skin bags, together with a statistical evaluation of the results. In the control preparations, which contained Ringer's solution on both sides, the electrolyte uptake was essentially the same for each individual animal. Addition of the solvent produced no significant changes.

Desoxycorticosterone, fluorohydrocortisone and hydrocortisone showed no influence when added in concentrations of 0.2 μg. per cc. In the higher concentrations of 2 and 20 μg. per cc. a considerable increase of the concentrating power of the skin bags was noted, as demonstrated by a pronounced

[2] We are indebted to Dr. Jay Gold for describing the details of this procedure. The solvent consisted of 30 parts propylene glycol to 2.5 parts benzyl alcohol to 0.5 parts Tween 80 to 100 mg. steroids. Stock solutions were then diluted with Ringer's to achieve desired concentrations.

difference between the untreated and treated preparations. In the experiments performed with 2 μg. of desoxycorticosterone great variations were noted, so that statistically the difference appeared insignificant ($P > 0.05$). In general, the effects of these steroids became more pronounced with higher concentrations. At a level of 2 μg. per cc., hydrocortisone and fluorohydrocortisone resulted in highly significant changes. At levels of 20 μg. per cc., both these steroids and desoxycorticosterone produced

TABLE 1. CHANGES IN MILLIOSMOLS PER CM.^{2} IN TREATED VS. UNTREATED FROG SKIN BAGS

Experimental conditions	Concentration μg./ml.	No. of observations	Mean change	Standard deviation	Standard error of mean
Controls	—	12	0.011	0.083	0.024
Controls with Solvent	—	12	0.015	0.089	0.027
Desoxycorticosterone	0.2	8	−0.053	0.096	0.036
	2.0	12	0.346	0.604	0.174
	20.0	12	0.538	0.470	0.135
Fluorohydrocortisone	0.2	8	−0.016	0.118	0.042
	2.0	12	0.489	0.189	0.055
	20.0	5	0.638	0.279	0.125
Hydrocortisone	0.2	8	−0.026	0.171	0.061
	2.0	8	0.619	0.280	0.099
	20.0	7	0.769	0.199	0.076
Estriol	2.0	5	0.001	0.024	0.017
	20.0	5	0.442	0.104	0.047
Testosterone	2.0	5	−0.054	0.088	0.040
	20.0	5	0.016	0.057	0.026

marked enhancement of salt transfer ($P \ll 0.01$). At the present moment however, it is not possible to state definitely to what extent these three substances differed in relative potency.

Testosterone in concentrations as high as 20 μg. per cc. had no effect. Estriol exhibited a statistically significant influence only in concentrations of 20 μg. per ml. No significant differences were noted in regard to the total fluid volume on the inside of the skin bags measured at the end of the experiments.

DISCUSSION

These experiments demonstrate that steroids affect the electrolyte transfer system by surviving isolated frog skin. Although only the osmolarity of the fluids was measured, it is probable that the changes are due primarily to sodium transfer. Previous observers have clearly shown that frog skin acts as a sodium pump. Moreover, sodium chloride predominated in the solutions used. It must be remembered, though, that these measurements taken after 16 hours represent the end state of an exchange, which consisted of a net influx of electrolytes, but which also undoubtedly in-

cluded a loss into the outside fluid. Experiments are being performed to study the effects of steroids at earlier time intervals and at different electrolyte concentrations. Although the changes expressed in milliosmols are highly significant, they actually represent relatively small quantities of electrolytes, which might well be missed when determined by standard chemical procedures. No effects on sodium transfer were observed by Huf and Wills (11) in animals pretreated with corticosteroids. No *in vitro* effects of steroids upon ion exchange have been demonstrated by previous investigations (11, 12), although, an influence of desoxycorticosterone upon electrical potentials of isolated frog skin was shown by Taylor and Weinstein (13). Recently, Meyers, *et al.* (12) have reported that ACTH administration to intact and hypophysectomized frogs increased the sodium transfer through the isolated skin. These results are compatible with those of the present experiments. The *in vitro* action of other hormones upon similar preparations have been demonstrated for epinephrine and Pitressin (14, 15).

Numerous observations on mammals and clinical experiments have shown that the sodium retaining power of steroids varies greatly depending on the steroid administered. Fluorohydrocortisone has a strong effect and resembles aldosterone. Desoxycorticosterone, although primarily a mineralo corticoid, has a much smaller effect. Hydrocortisone is still weaker in this respect, while estrogens are less potent, and androgens are least effective. In the present investigation on frog skin, such quantitative gradients were suggested, but were not proven. Unfortunately, no aldosterone was available for testing. Interestingly, estriol acted only in higher concentrations, and testosterone was ineffective. No definite conclusions in this regard can be drawn because no data exist in respect to the differential action of these substances in whole amphibians. Other organs, such as the kidney, may determine the ultimate status of electrolyte balance. The quantitative effects of the various steroids may be different when exerted on the kidney than when acting at extra-renal target structures.

The present experiments have demonstrated that steroids act directly on the frog skin. Linderholm (9) and Huf (7) have pointed to the importance of the basement membrane in the transfer of sodium. Recent investigations have suggested that electrolytes may be bound by the mucopolysaccharides of the intercellular substance, and alterations of this structure may bring about a change in their content (16, 17). It has been repeatedly suggested that steroids affect the structure of this same substance (18, 19). Whether the effects upon sodium transfer are related to this particular property remains to be proven.

SUMMARY

Effects of steroids on the electrolyte transfer of isolated frog skin were studied. Skin bags of hind limbs were suspended in Ringer's solution with the epithelial surface remaining on the outside. Various steroids added to

the Ringer's solution were placed on the inside of the skin bag from one limb, while the other contained Ringer's solution alone and served as a control. Osmotic pressures of the inside and outside fluids were examined at the beginning and the end of each experiment. In control experiments in which Ringer's solution only was present, or in which the solvent alone was added, no significant difference existed between the uptake of electrolytes in the preparations from each individual animal. Desoxycorticosterone, fluorohydrocortisone and hydrocortisone caused a considerable increase in the rate of transfer of electrolytes to the interior of the frog skin bags as determined by osmotic pressure changes during incubation. Estriol showed an effect only in higher concentrations. Testosterone exhibited no effect. The significance of these findings was discussed.

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EFFECT OF ESTROGEN ON THE METABOLISM OF PROTEIN-BOUND IODINE*†

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A RELATIONSHIP between gonadal and thyroidal functions has long been suspected and frequently studied, with conflicting or inconclusive results. The present investigation was, therefore, initiated to examine more thoroughly the effect of estrogen on thyroidal function and protein-bound iodine. The results indicated that estrogen increased the protein-bound iodine content of the thyroid and its hormonal turnover.

MATERIALS AND METHODS

Male rats (Holtzman) were castrated 10 to 20 days before use for these studies. Four experiments were done.

I. Experimental groups received 500 I.U. of water-soluble estrone[1] subcutaneously daily for 4, 16 and 31 days, the dose being contained in 0.2 ml. of saline. Controls were given 0.2 ml. of saline by the same route over the same period of time and were run simultaneously with their corresponding experimental animals. On the day of the last estrogen or saline injection I^{131} was administered intraperitoneally. Forty-eight hours later the animals were sacrificed by etherization and exsanguination. The thyroids were immediately dissected out, cleaned of adventitial tissue and weighed on a Roller-Smith torsion balance to the nearest 0.1 mg. Total radioactivity in the glands was recorded in a well-type scintillation counter. The thyroids were then homogenized in 5 ml. of doubly distilled water. An aliquot of the homogenate was precipitated by Somogyi reagents, washed four times with 0.1% KI solution, and the radioactivity in the precipitate recorded. The remainder of the homogenate was analyzed chemically for protein bound iodine (PBI) according to Danowski *et al.* (1). Sera were handled in a similar manner. The total radioactivity in an aliquot was recorded and the aliquot was treated for the determination of protein-bound I^{131} (PBI^{131}) as described above for thyroid homogenates. The remaining serum of each animal was used for chemical determination of PBI.

II. Thirty-eight rats were divided into 6 experimental groups of 5 each and a control of 8 animals. Each experimental rat received subcutaneously a total of 10,000 I.U. of estrogen contained in 0.2 ml. of saline according to the following schedule: Group 1, daily doses of 2,000 I.U. for 5 days; Group 2, daily doses of 2,500 I.U., for 4 days; Group 3, daily doses of 3,333 I.U. for 3 days; Group 4, daily doses of 5,000 I.U. for 2 days; Groups 5 and 6, one injection of 10,000 I.U. Groups 1 to 4 were given I^{131} intraperitoneally immediately after the last injection of estrogen; Group 5 received I^{131} and estrogen at the

Received June 5, 1956.

* This work was supported by grant (C-2579) from the National Cancer Institute, and by grant A-456 (C5) from the Arthritis and Metabolic Diseases Institute, National Institutes of Health.

† This is paper #62 from the Department of Pathology, University of Pittsburgh School of Medicine.

[1] Theelin, Parke, Davis and Co. water-soluble estrone, 50,000 I.U. per cc.

same time; Group 6 received I^{131} 18 hours before the administration of estrogen. Controls were injected with 0.2 ml. of saline for from 1 to 5 days and received I^{131} immediately after the last saline injection. All rats were sacrificed 24 hours after the administration of I^{131} and were treated as the animals of the previous experiment.

III. Twenty-eight rats were divided into 4 groups of 7 each. 250 I.U. of estrogen in 0.2 ml. of saline were administered subcutaneously to two groups daily throughout the experiment, beginning two days before the administration of I^{131}. Controls received 0.2 ml. of saline. Twenty μc. of I^{131} were given intraperitoneally. Daily *in vivo* thyroidal counting, under light nembutal anesthesia, was begun 40 hours later and continued for 5 days. After the first thyroid counting, one estrogen-treated and one control group were injected daily with 2 mg. of tapazole[2] subcutaneously.

IV. Fourteen rats were put on a low iodine diet (Remington)[3] beginning the day of castration and continued for the duration of the experiment. Eleven days later they were injected with 30 μc. of I^{131} and placed in individual metabolism cages. Two days after the administration of I^{131} 7 animals were injected with 250 I.U. of estrogen and daily thereafter for the duration of the experiment. At this time also, i.e., two days after the I^{131} injection, all animals were given 250 μg. of sodium iodide per 100 gm. of body weight daily subcutaneously, to reduce the recycling of I^{131} from degraded PBI^{131} (2, 3, 4). Urine and feces were collected at 24 hour intervals and radioactivity in the excreta was determined. Thyroid radioactivity was recorded at the same time daily under light nembutal anesthesia; corrections were made for body background and decay. Tail vein blood was removed daily at the same time each day and serum radioactivity recorded. PBI^{131} in sera was determined by the method described above. The animals were sacrificed 8 days after the administration of I^{131}. Their thyroids and sera were treated as in the previous experiments.

Total radioactivity remaining in the animals was recorded by dissecting the rats and counting their parts, after removal of the bladder and thyroid, in a Texas G-M counter.

The I^{131} was obtained from Oak Ridge National Laboratories, Oak Ridge, Tennessee. Duplicate standards, appropriately diluted, were prepared from the same volume of I^{131} that was injected into the animals. Decay corrections and microcurie dose were calculated from these standards.

For counting, a thallium-activated NaI well-type crystal was used with a sensitivity of 1×10^6 c/m per μc. of I^{131}. Counting rates were such that the sampling error was less than 2%. Feces and animal tissues were counted in a G-M Texas counter with a sensitivity 1/15 that of the crystal counter.

Calculations for the above experiments were as follows. Total thyroidal radioactivity per 100 mg. of thyroid tissue and serum PBI^{131} per 100 ml. of serum were expressed as per cent of dose. Thyroidal PBI^{131} was calculated as per cent of total thyroidal radioactivity. Thyroidal and serum PBI were calculated as micrograms per 100 mg. of thyroid tissue and per 100 ml. of serum respectively. Radioactivity decay in the thyroid and serum was plotted on semilog paper against time and the half-life determined by inspection of the curves obtained. Daily proportional turnover rate was derived from the formula $.693/t_{\frac{1}{2}}$. The proportion of total thyroidal PBI turned over daily was obtained by multiplying the total thyroidal PBI by this factor.

RESULTS

In Table 1 the data of Experiment I are presented. The pertinent observations were as follows:

1. The administration of estrogen over 3, 15 and 30 days significantly

[2] Tapazole (Methimazole) was generously supplied by Dr. C. G. Weigand, Eli Lilly and Co., Indianapolis, Indiana.

[3] Prepared commercially by Nutritional Biochemicals Corp., Cleveland, Ohio.

TABLE 1. EFFECT OF ESTROGEN ON THYROIDAL AND SERUM PBI^{131} AND PBI

Animal	No. of rats	Body wt., gm.	Thyroid			Serum		
			Thyroid wt., gm.	I^{131}/100 mg. % of dose	PBI^{131} / Total I^{131}	PBI, μg./100 mg.	PBI^{131}/100 ml. % of dose	PBI, μg./100 ml.
Estrogen 3 days	7	177	9.3	*139 ± 8.1**	89	†*76.6 ± 2.7*	*4.2 ± .36**	4.2
Control	7	214	8.9	58 ± 5.5	89	66.2 ± 3.2	2.3 ± .14	4.1
Estrogen 15 days	7	188	11.0	*71 ± 3.1*	93	94.2 ± 2.5	1.6 ± .11	3.5
Control	7	246	11.0	50 ± 3.7	97	88.6 ± 5.0	1.6 ± .11	3.4
Estrogen 30 days	7	247	11.1	*91 ± 4.8*	79	86.3 ± 5.0	2.7 ± .19	3.4
Control	7	323	12.5	65 ± 4.9	79	77.0 ± 3.7	1.8 ± .18	3.4

* Standard error of mean.
Italicized numbers significantly different from controls at 1% level.
† P = .02.

increased the accumulation of thyroidal I^{131} compared with controls, the maximum being obtained after 3 days of estrogen injections.

2. The proportion of thyroidal I^{131} converted to PBI^{131} was approximately the same in both estrogen-treated and control animals.

3. Thyroidal PBI was greater in estrogen treated rats than in controls, significantly so in the group receiving estrogen for 3 days.

4. The concentration of serum PBI^{131} was significantly greater in animals treated with estrogen for 3 and 30 days.

5. Chemical analysis of serum PBI revealed no significant differences between experimental and control groups.

6. Body weight of estrogen treated rats was less than that of controls. Thyroid weights were not significantly affected by the administration of the ovarian hormone. However, if thyroid weights were computed on the basis of body weight, some of the estrogen-treated groups would have shown a significant increase in thyroid/body ratio.

The results of Experiment II are shown in Table 2. It was obvious that the length of time during which the same large dose of estrogen was administered significantly enhanced the accumulation of thyroidal I^{131} and PBI^{131}. Serum PBI^{131} was also elevated by the injection of the ovarian hormone, in proportion to the period of administration. Thyroidal PBI was significantly

TABLE 2. EFFECT OF ESTROGEN AND TIME ON THYROIDAL AND SERUM PBI^{131} AND PBI

	Animal	No. of rats	Body wt., gm.	Thyroid			Serum		
				Thyroid wt., mg.	I^{131}/100 mg. % of dose	PBI^{131} / Total I^{131}	PBI, μg./100 mg.	PBI^{131}/100 ml. % of dose	PBI, μg./100 ml.
Estrogen	5 days	5	287	14.3	153 ± 10.7	75	80.6 ± 5.9	2.86 ± .52	3.2
	4 days	5	180	12.6	108 ± 11.0	78	82.5 ± 3.8	2.12 ± .10	2.9
	3 days	5	185	13.5	113 ± 1.3	81	96.5 ± 4.3	2.18 ± .16	3.2
	2 days	5	197	12.9	78 ± 6.5	78	93.2 ± 10.1	1.83 ± .17	3.3
	1 day	5	198	12.2	68 ± 3.8	72	65.0 ± 6.5	1.78 ± .14	3.2
	½ day	5	211	12.3	62 ± 5.6	71	79.4 ± 5.4	1.66 ± .06	2.9
	Control	8	239	13.7	59 ± 3.0	78	61.4 ± 3.0	1.20 ± .10	3.7

elevated in rats treated with estrogen for two or more days, as compared with control groups. Serum PBI was not significantly affected by estrogen.

The results of Experiments III and IV are graphically presented in Figures 1 and 2 respectively. The administration of estrogen to castrate male rats did not influence the rate of loss of thyroidal radioactivity nor the

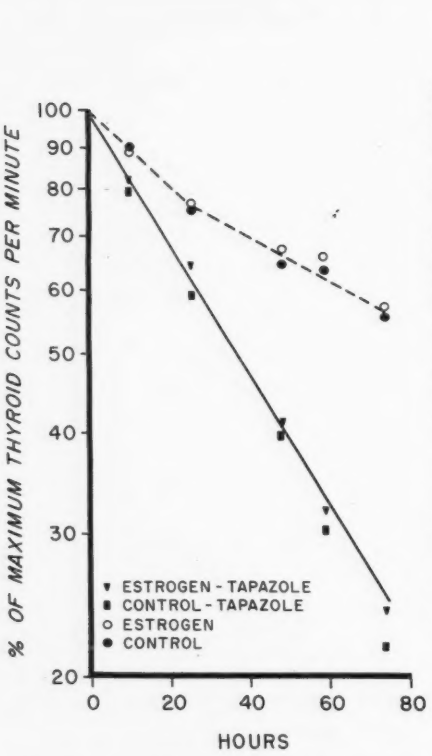

FIG. 1. Effect of estrogen on radioactive decay in the thyroid after administration of I^{131}, with and without tapazole.

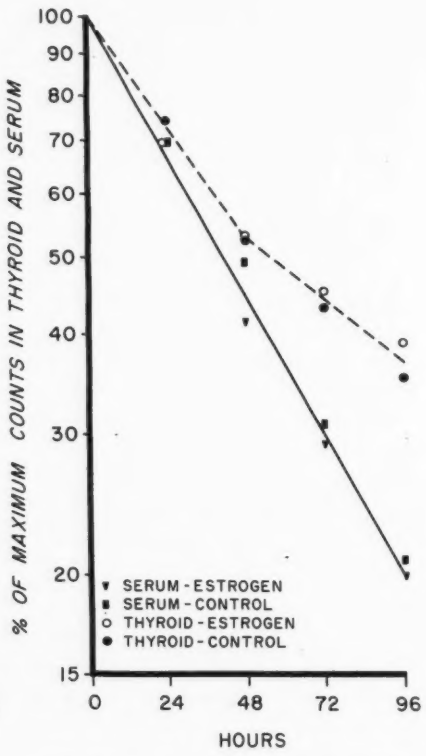

FIG. 2. Effect of estrogen on radioactive decay in the thyroid and serum after administration of I^{131}.

rate of decline of serum PBI^{131} (Figure 1). When tapazole was given to prevent recycling of I^{131}, radioactivity in the thyroid was lost at a constant rate with a half-life of 42 hours. Without the use of a blocking agent the plot of radioactive decay in the thyroid against time did not form a straight line. Most likely this was due to recycling of I^{131}.

In Figure 2 the decline of serum PBI^{131} was linear on a semi-log plot with a half-life of 37 hours. Decline of radioactivity in the thyroid was again diphasic, and it was apparent that thyroidal loss of I^{131} during the first few days was at approximately the same rate as the decline of serum PBI^{131}.

In Table 3 are shown the values for PBI secretion in the experimental and control groups of rats of Experiment I. The total quantity of PBI turned over each 24 hours was calculated from the total thyroidal PBI and the proportional turnover rate of 41.5% per day. Rats receiving estrogen over 3 and 15 days degraded more PBI daily than their controls. When these values were calculated on the basis of body weight, the daily quantity of PBI turned over was approximately 25 to 45% greater in all the experimental groups than in the controls.

TABLE 3. EFFECT OF ESTROGEN ON THYROIDAL PBI TURNOVER

Animal	Total PBI turnover/day, μg.	PBI turnover/day per 100 gm. body wt., μg.
Estrogen 3 days	3.24	1.83
Control	2.82	1.31
Estrogen 15 days	4.27	2.27
Control	3.82	1.55
Estrogen 30 days	3.94	1.59
Control	4.02	1.25

DISCUSSION

These data taken together indicated that estrogen enhanced the thyroidal accumulation of I^{131}, PBI^{131} and PBI, and also increased the quantity of PBI turned over daily.

There has been no unanimity concerning the effect of estrogen on thyroidal accumulation of I^{131}. In mice and rats estrus and estrogen have been reported to increase thyroidal I^{131} (5, 6, 7). Others (8, 9) have reported that small doses of estrogen increased thyroidal uptake of I^{131} and large doses depressed thyroidal accumulation of the isotope. Money *et al.* (10) stated that different estrogens enhanced or suppressed thyroidal accumulation of I^{131}. Paschkis, Cantarow and Peacock (11) were unable to observe any effect of estrogen on thyroidal uptake of I^{131}. Pochin (12) studied thyroidal accumulation of I^{131} in women during the menstrual cycle and did not observe any appreciable variation in I^{131} uptake during different stages of the cycle. Nor did Zingg and Perry note any consistent action of estradiol on iodine metabolism (13). Some of these negative results might be explained by the fact that the administration to rats of moderate doses of estrogen for 15 to 30 days tended to decrease thyroidal uptake of radioactive iodine (7). In the present investigation, estrone in comparable or larger doses consistently enhanced the thyroidal content of I^{131}.

Quantitative determinations of thyroidal iodine content were made by Koenig *et al.* (14) and Gassner *et al.* (15). In their reports the administration of the ovarian hormone for 12 weeks markedly reduced the iodine content

of the gland. Our experiments have shown repeatedly that the injection of estrogen in similar amounts up to 30 days increased the PBI of the thyroid. In unpublished work (16), estrogen was given to rats for 15 months and no change in thyroidal PBI content was noted compared with controls.

The half-life of thyroidal radioactive iodine, measured *in vivo* without the use of blocking agents, has ranged from two to eight days, depending on the conditions of the experiments (3, 17, 18, 19). This measure of thyroidal decay is not one of simple secretion or loss but represents both reaccumulation of I^{131} from degraded PBI^{131} and discharge of PBI^{131} from the gland. In the rat, the proportion of PBI^{131} turned over daily is quite high and significant quantities of I^{131} can be recycled and accumulated by the thyroid. To obtain a more accurate measurement of thyroidal loss of I^{131}, recycling should be prevented. When tapazole was given as a blocking agent to rats injected with I^{131}, the thyroidal decay of radioactivity showed a half-life of 42 hours in both estrogen-treated and control animals. Without a blocking agent, thyroidal half-life of I^{131} would have been 3.5 days or more if a straight line were assumed to pass through the plotted points of Figure 1. Albert and Tenney (18) found a half-life of 24 hours using propylthiouracil and a half-life of 8 days without the blocking agent. They suggested that propylthiouracil accelerated the discharge of thyroxine from the thyroid.

The thyroidal I^{131} half-life of 42 hours with tapazole was very close to the half-life of serum PBI^{131}, which was 37 hours. Thyroidal and serum decay was the same in both experimental and control animals. The almost equal rates of decline in thyroidal and serum radioactivity indicated a steady state with respect to PBI^{131}. Such a steady state has been shown to prevail in rats (20) and it was reasonable to assume that the control rats of this experiment were in equilibrium with respect to iodine. Radiothyroxine half-life in rats has been reported to vary from 15 to 18 hours, a distinctly faster decay rate than reported here (21, 22, 23). However, these rates were based on the administration of exogenous thyroxine, and there is evidence that the rat degrades exogenous thyroxine faster than endogenous thyroxine (16).

The quantity of PBI turned over daily, per 100 gm. of body weight, ranged from 1.25 μg. to 1.55 μg. in controls and from 1.59 μg. to 2.27 μg. in estrogen-treated animals. The quantity of thyroxine secreted daily in rats has been determined by a number of investigators and has ranged from 1.4 μg. per 100 gm. of body weight to 10 μg. (24–28). In more recent studies the best estimates have been from 1.4–2.0 μg. per 100 gm. of body weight (25, 27, 28). The present results agree well with these latter estimations.

Since there was no rise in serum PBI nor an increased serum PBI turnover rate and since there was greater discharge of thyroidal PBI in estrogen-treated rats, the experimental animals either utilized or degraded

the increased quantity of PBI or the PBI space of distribution was enlarged in these animals. Unfortunately the calculation of PBI space (radioactivity remaining in the body as PBI^{131} divided by the concentration of PBI^{131} in one ml. of serum) is subject to large error. In unpublished studies (16), rats were sacrificed at varying time intervals after the administration of a known amount of I^{131}. Fecal and urinary radioactivity were determined and also the total remaining in the body. The best recovery after 3 days was 87% of the dose injected. A loss of 13% of the injected dose was large in comparison with the concentration of radioactivity in the serum. Calculation of PBI^{131} space without cognizance of this loss could expand the space several-fold. With knowledge of this source of error in mind, PBI^{131} spaces were calculated for both estrogen treated and control rats of experiment IV, and expressed as a percentage of body weight, for each day of the study. PBI^{131} space was greater in the control rats than in the experimental animals. The calculated space increased from day 1 to day 5 of the experiment and ranged from 144% to 653% in controls and from 112% to 378% in estrogen treated rats. If correction was made for radioactivity which was not recovered in feces, urine and body tissues, then the PBI^{131} spaces were almost the same in the two groups of rats. In controls this corrected space ranged from 67% to 150% (day 1 to day 5) and from 70% to 128% in estrogen-treated animals.

Serum PBI^{131} but not PBI, was usually higher in estrogen treated rats than in controls. On the other hand, Engstrom and Markardt (29) demonstrated a significant elevation of serum PBI in human subjects given stilbesterol. Similar elevations of serum PBI have been noted during pregnancy, although it has not been determined whether this is due to estrogen or other causes (30, 31). The shift of serum PBI during pregnancy recently described by Robbins (32) and by Dowling and his associates (33) might explain this phenomenon. The elevated serum PBI^{131} in the experimental rats indicated thyroidal secretion of PBI with a higher specific radioactivity than that in control rats. As the data of this paper have shown, there was also increased secretion of thyroidal PBI and the increased quantity degraded at the same rate, so that serum PBI levels remained unchanged. Other investigators, employing other methods, have shown indirectly that the administration of estrogen increased the quantity of thyroxine degraded daily. Sherwood and his colleagues, in a series of publications, reported that in rabbits and rats made hyperthyroid by desiccated thyroid the injection of estrogen rapidly eliminated the hyperthyroid state (34, 35, 36). In thyroidectomized rats fed desiccated thyroid, the ovarian hormone lowered the BMR to hypothyroid levels in half the time required for control rats to reach the same hypothyroid state (37, 38). Gessler demonstrated a similar effect of estrogen on the hypothyroid state of guinea pigs (39). Schultze and Turner performed extensive studies in chicks employing the goiter production assay method (40). They found that in several strains of

chicks, the injection of the proper doses of estrogen increased the thyroxine requirements for the prevention of thiouracil-induced goiter. Desclin and Ermans in rats reported similar results (41). In the papers just cited, several postulates were offered to explain the results: 1) that estrogen neutralized thyroxine (34–38); 2) that estrogen inhibited hypophyseal TSH and consequently inhibited the production and discharge of thyroxine (39); 3) that estrogen in proper dosage stimulated hypophyseal discharge of TSH, thyroxine discharge and utilization (40, 41). The present experiments were not designed to test these hypotheses but they have corroborated their findings, namely, that more thyroxine was turned over daily in the rats given estrogen.

Whether the administration of estrogen induced a hypermetabolic state was not ascertained. Despite the evidence of increased turnover of PBI there was no firm evidence that the ovarian hormone elicited hyperthyroidism. This was in accord with what has been found during pregnancy, when serum PBI was elevated and symptoms and signs of hyperthyroidism were lacking. The animals did lose weight, however, when given estrogen for as short a period as 3 days. It was not established whether this weight loss was the result of increased thyroidal secretion of PBI, diminished food intake, or some other action of estrogen on body metabolism.

SUMMARY

The administration of estrogen to castrate male rats increased thyroidal I^{131}, PBI^{131} and PBI. Serum PBI^{131} was also elevated and serum PBI remained unaltered. Estrogen did not change the rate of thyroidal or serum PBI^{131} decay. However, in estrogen treated animals the daily quantity of PBI secreted by the thyroid and degraded peripherally was greater than in control animals.

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LOSS OF APPETITE FOR ALCOHOL AND ALCOHOLIC BEVERAGES PRODUCED IN RATS BY TREATMENT WITH THYROID PREPARATIONS

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SELF-SELECTION experiments have shown that homeostasis of an organism is maintained not only by the physiological regulators as described by Claude Bernard (1) and Walter Cannon (2), but also by behavioral regulators that are manifested in the activities of the total organism (3). In the normal organism both forms of regulation operate toward the maintenance of homeostasis and when one form of regulation ceases to function the other takes over. For example, after the physiological regulator for sodium metabolism has been eliminated by removal of the adrenal glands and life is threatened by the excessive loss of sodium in the urine, the animal eats or drinks more salt, enough to compensate for the loss. Many other instances of this kind could be cited.

In the common laboratory rat appetite has proved to be such a reliable guide to dietary needs that it can be used as a tool for the study of the nutritive value of food-stuffs and of the factors involved in their utilization as well as an indicator of the specific mechanisms involved in the functioning of the various endocrine glands.

The mechanisms involved in the operation of behavioral dietary regulations of rats are known at least in part. Taste plays an important part since section of the taste nerves seems to abolish the ability to regulate the intake of nutritive and non-nutritive substances; furthermore, so far no instances have been found in which rats are able to make beneficial selections or avoid injurious ones from substances having no taste. Thus, poisons having no taste are readily accepted in lethal amounts although poisons with a taste are rejected in even minute amounts (4). How these behavioral mechanisms can be broken down to result in faulty choices has also been studied.

Observations on dietary selections of the rat are of special interest since the rat's dietary needs closely resemble those of man; as a consequence much of our knowledge about man's dietary needs comes from observations on the rat.

It is of interest therefore to learn how the rat reacts to alcohol, whether it regards alcohol as a food or a poison; as well as whether and how its

Received June 5, 1956.

ability to regulate its intake of alcohol can be broken down to result in excessive and harmful use of alcohol.

Our first step was to determine whether the rat can taste alcohol and in what concentration—and whether it regards it as a food or a poison. For this purpose we used our so-called taste preference threshold technique (3). Each rat was housed in a cage containing two graduated 100 ml. bottles for fluids and a food-cup. Food and fluid intake were recorded daily. At the start both bottles were filled with distilled water and intake records were taken for several weeks to get base-lines. Then one bottle was filled with a subliminal concentration of alcohol and each day the concentration was

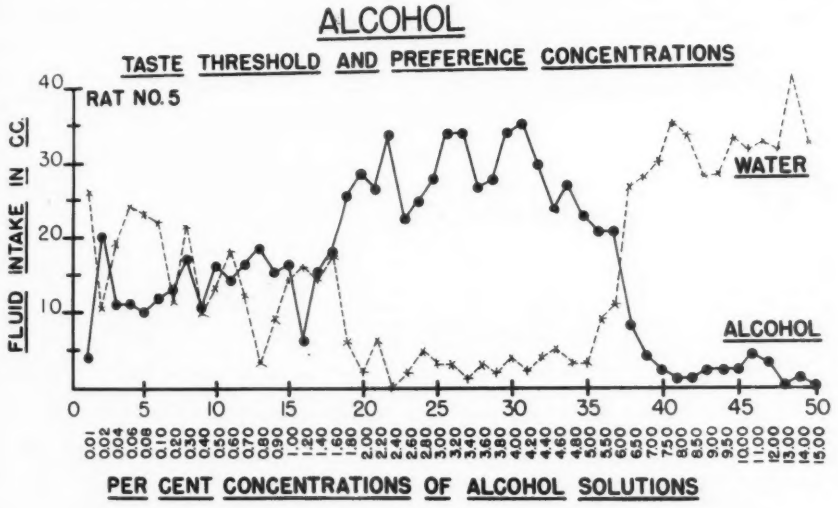

FIG. 1. Alcohol taste preference threshold curve for a normal rat.

increased in small steps until the rat showed a consistent preference for either the alcohol solution or water (5). Figure 1 shows a record for one rat. For 18 days, until the concentration of alcohol reached 1.4% the rat continued to take equal amounts of the water and the alcohol solution. At this point it started to take more of the alcohol solution and less water until a plateau was reached and maintained between 1.8 and 6%. After that it began to drink less alcohol and satisfied its thirst with water. Rats show a considerable amount of variation in these taste threshhold curves.

From previous experimental experience the preference of alcohol solution over water indicates that rats regard alcohol as a nutritive substance and not as a poison. Thus, they show similar preferences for various needed mineral solutions, for sugars and for vitamins (3); on the other hand they show a preference for water over infinitesimally low concentrations of poisons (4).

Further evidence that rats regard alcohol as a food comes from experiments in which fluid intake was restricted to an 8, 16, or 24% solution of alcohol, so that the rats were forced to take a certain amount of alcohol in order to satisfy their thirst (6).

Under these circumstances the rats reduced their food intake in direct proportion to the calories received from the forced ingestion of alcohol. The total caloric intake remained unchanged.

This was found to be true also when the rats had to satisfy their thirst from wine, beer, or whiskey as a solution of 12% alcohol by volume (7). Figure 2 shows a typical record for a rat with access to beer.

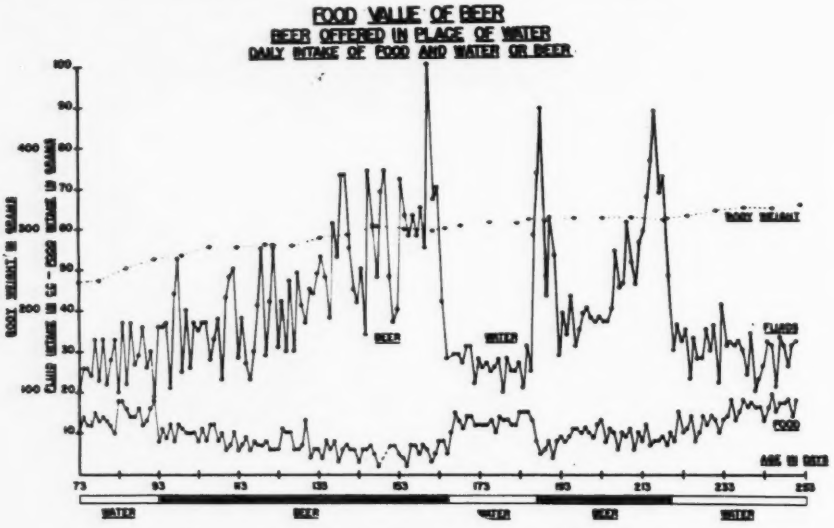

FIG. 2. Graph showing daily intake of food, water or beer in a rat restricted either to beer or water.

The food value of alcohol was demonstrated in the alcohol experiments by the observation that although some rats on the 16 and 24% concentrations derived 40–50% of their total caloric intake from alcohol over periods of 10–15 months, they remained in excellent shape and in fact were some of the healthiest looking animals that we have had.

Thus, so far we have not succeeded in making rats take more than a certain total number of calories—so long as they are given the opportunity to regulate their intake. That they do maintain a constant total caloric intake is a cardinal principle of dietary self-regulation. This holds also for substances other than alcohol: sugars, fats or oils.

That man gets into difficulties with alcohol may result from his failure to recognize that alcohol has a high caloric value and is an excellent food; also from his credo that ingested alcohol needs a lot of food to sop it up, so

to speak, and which results in the ingestion of far more calories than can burned effectively if at all.

Further experiments were undertaken to determine whether by prolonged forced feeding of alcohol rats could be made to develop an abnormal appetite for alcohol, a craving that might ultimately lead to their undoing (8). These rats were forced to take 8, 16 and 24% solutions of alcohol as the sole source of fluid over long periods of time, 18 months in some instances. At the end of this time when given access to water the rats gave no definite indication of having become addicted to alcohol. These experiments were repeated a number of times always with the same results.

It still seemed possible that an alcohol addiction might be produced in this way by forcing the rats to drink even larger amounts of alcohol, and to take even smaller amounts of food and water.

Such an opportunity seemed to be offered by the results of some other experiments in which it was found that rats treated with thyroid powder

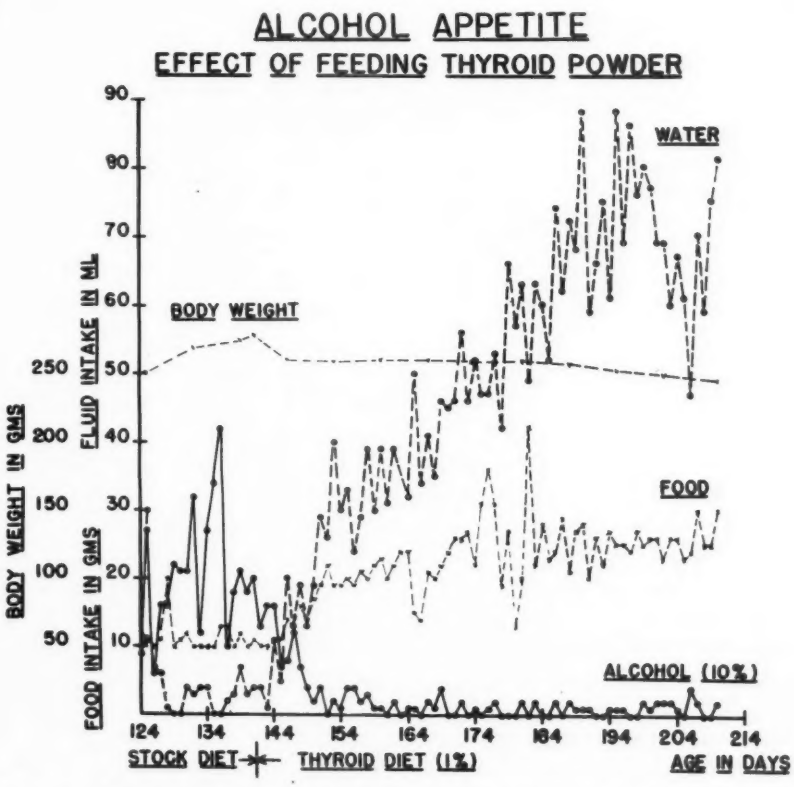

FIG. 3. Graph showing daily intake of water, food and an alcohol solution (10%) before and after addition of thyroid power (1%) to the normal diet.

greatly increased their intake of sugar solution and decreased their intake of food and water. We decided to try to produce a high alcohol intake by making the rats hyperthyroid.

Here we were in for a surprise as may be seen in Figure 3 which gives the intake of water and a 10% solution of alcohol in milliliters and food intake in grams on the ordinates; and time in days on the abscissas. Before the start of the thyroid feeding this rat drank about 15 ml. per day of the alcohol solution and only small amounts of water. Almost immediately after thyroid powder in a 1% concentration was added to the food the rat

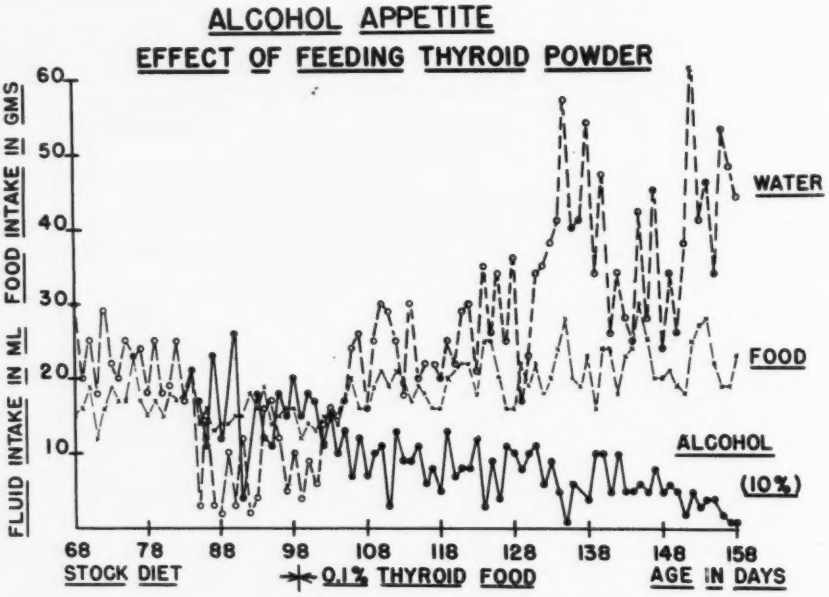

FIG. 4. Same as Fig. 3 except for the percentage (0.1%) of thyroid powder in the diet.

stopped drinking the alcohol solution, drank much more water and ate more food. Figure 4 shows that on a 0.1% concentration of thyroid powder another rat gradually reduced its alcohol intake to zero but over a longer period. Concentrations as low as 0.04% were effective in reducing the alcohol intake.

Feeding thyroid powder had the same effect on the appetite for wine and beer and whiskey. See Figures 5 and 6. Other thyroid preparations, thyroxine and triiodothyronine were equally effective.

Rats made hypothyroid by removal of the gland may develop an increased appetite for alcohol—that is simply a preference of the alcohol solution over the stock diet. A total of 60 rats were used for the experiment on the relation of alcohol intake to thyroid state or function.

Thus, by its appetite the rat indicated a relationship between thyroid

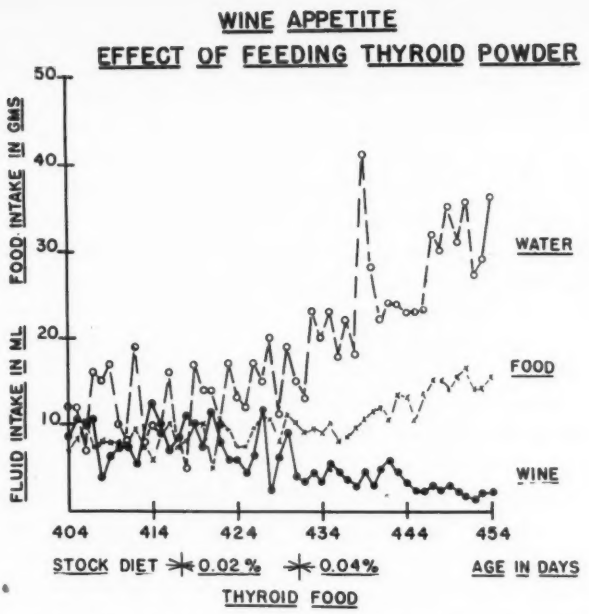

FIG. 5. Graph showing daily intake of water, food and wine (common table wine) for a normal rat before and after the addition of thyroid powder (0.02 and 0.04%) to the stock diet.

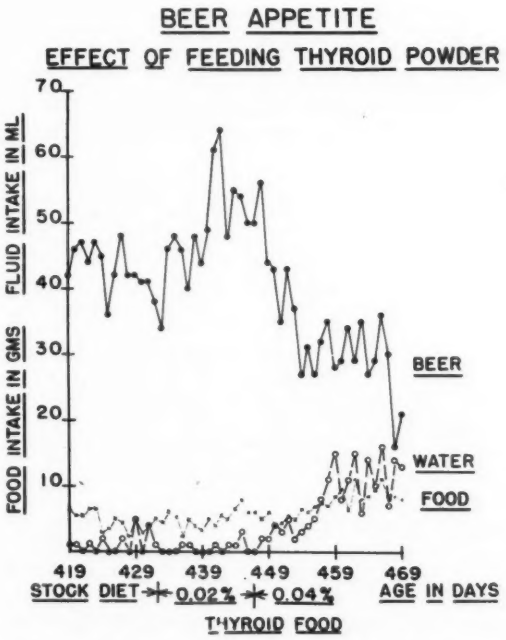

FIG. 6. Graph showing daily intake of beer, water and food of a normal rat before and after the addition of thyroid powder (0.02 and 0.04%) to the normal diet.

metabolism and the appetite for alcohol. That the hyperthyroid rat reduces its intake of alcohol or refuses to drink it altogether, indicates to us that it cannot use alcohol. Actually forced feeding of hyperthyroid rats with alcohol produced demonstrable lesions in the adrenals and kidneys.

At this point it became of interest to determine to what extent a similar relationship exists in man. Inquiries were made among medical men and surgeons to determine how often hyperthyroids are alcoholics; how often alcoholics are hyperthyroids. It was found that hyperthyroids are rarely ever alcoholics; in fact one surgeon who had treated many hyperthyroids had not seen one that he thought might be alcoholic. Likewise it was the general opinion that alcoholics who are hyperthyroids are rare.

It might follow then that in man treatment with thyroid preparations might help alcoholics through reducing their appetite for alcohol.

One would assume that alcoholics are hypothyroid at least to some degree. They may have originally become hypothyroid in acting on the aforementioned credo—the necessity of eating food to sop up ingested alcohol—thereby ingesting far more calories than can be utilized effectively. It is possible that under these conditions the thyroid cannot produce enough thyroxine to metabolize the excessive intake of calories and so that there is a constant deficit.

The results of other experiments showed that a thyroid deficiency may possibly result from permanent damage to the thyrotropic cells of the pituitary or to the thyroid stimulating cells in the hypothalamus—that injury to these structures might have to be taken into account in the study of alcoholism (9).

Whether the rat's appetite will actually be a useful guide in working out the problem I will leave to clinicians who are actively engaged in treating alcoholics.

SUMMARY

It was found that rats made hyperthyroid have a decreased appetite for alcohol, an increased appetite for the stock diet and sugars, and an increased thirst for plain water.

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THE INFLUENCE OF ANDROGEN ON THE MALE ACCESSORY REPRODUCTIVE GLANDS OF THE GUINEA PIG: STUDIES ON GROWTH, HISTOLOGICAL STRUCTURE AND FRUCTOSE AND CITRIC ACID SECRETION[1,2]

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EXTENSIVE studies have been made in the past on the accessory reproductive glands of the guinea pig. Sayles (1, 2) observed that in this species, histological differentiation of the glands of young males was not completely inhibited by castration, and that regression of the glands of adult castrates did not occur in the sudden and striking manner that is typical of the glands of the rat. These findings raised the question of the relation of androgen to differentiation and to functional activity and suggested that a certain degree of normal secretory activity might proceed in the glands in the absence of testicular androgen.

The important discovery of fructose and citric acid in the accessory glands of several species and the demonstration that the secretion of these substances is dependent on androgen (3, 4, 5, 6, 7, 8) have made available clear-cut criteria for determining the relation of androgen to the development and maintenance of specific functional activity. Some studies on secretion of fructose and citric acid have been made in the guinea pig. Humphrey and Robertson (9) have shown that in the normal adult, fructose and citric acid are secreted by the seminal vesicle, while other glands, which they designate as dorsal and ventral prostates, produce only citric acid. Further investigations of the effect of androgen on seminal vesicle fructose were made by Levey and Szego (10).

The work to be presented here is a study of the influence of androgen on the development and maintenance of four accessory glands of the guinea pig, as determined by fructose and/or citric acid secretion, weights and histological structure. Preliminary reports have appeared on parts of this work (11, 12).

Received May 11, 1956.

[1] This research was aided by Grant G2912 United States Public Health Service and by the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

[2] The authors are indebted to Miss Frances Daner for technical assistance.

[3] Scholar in Cancer Research of the American Cancer Society.

MATERIALS AND METHODS

The seminal vesicles, the lateral and dorsal prostates and the coagulating glands were studied. The glands were classified as in Figure 1, which follows Walker (13) for the seminal vesicle and the coagulating gland. In the present classification, however, the dorsal prostate corresponds to the posterior lobe, while the lateral prostate is equivalent to the anterior lobe in his classification. It was considered advisable to separate the prostate into two components, in view of their embryological origin as two independent primordia, each of which joins the prostatic sulcus by a single duct (14).

Studies were made on 152 males, which included normal animals from birth to 180 days of age (Table 1), prepuberal and adult castrates with and without androgen treatment and androgen-injected intact young (Table 2). Prepuberal castration was done either at birth or at 10 days of age, and adults were castrated at 120 days. Testosterone propionate[4] was used, and each dose was injected subcutaneously in 0.2 cc. sesame oil.

After castration at birth some of the young remained untreated, while the others received 1 mg./day of the androgen; they were all autopsied at the age of 28 days. In addition, the same dose was given to normal young from birth to 10 days or from birth to 28 days. When males were castrated at 10 days, some were autopsied at 24 days (after two weeks of castration) and others at 40 days (after four weeks of castration). In the 40-day-old group, some had received 2.5 mg./day of androgen during the two weeks prior to autopsy.

Adult castrates were autopsied 4 weeks or 8 weeks after the operation. Among the 8-week castrates some received a daily dose of 2.5 mg. of androgen during the last two weeks of life.

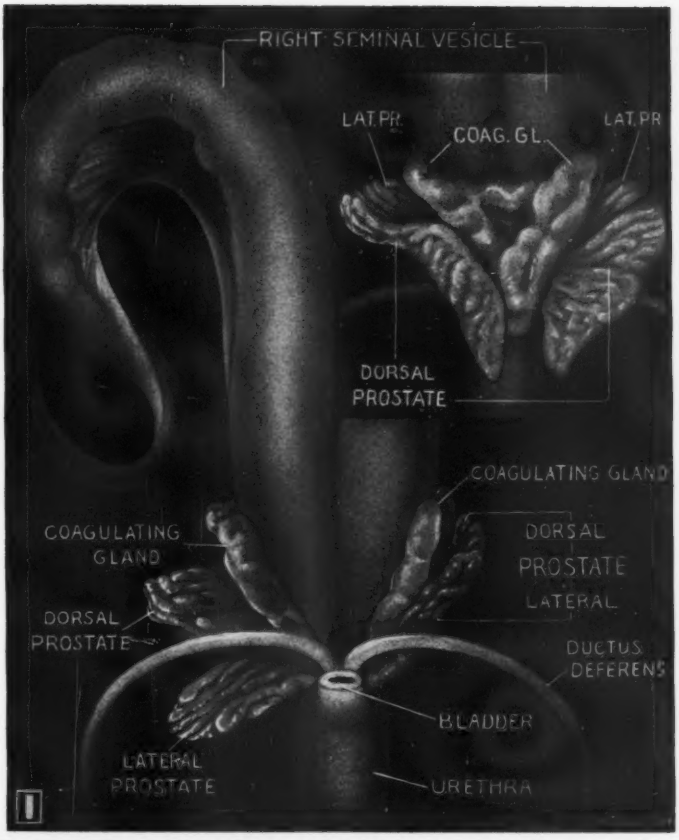
The animals were autopsied after slight ether anesthesia followed by decapitation and bleeding from the neck. The glands were dissected and removed immediately, taking special care to avoid contamination of one gland with the secretion of the others. Each gland was weighed fresh, a small piece was fixed in Bouin's fluid for histological study, and the remainder was used for chemical analysis. Sections were cut at 7 μ. and stained in Harris hematoxylin. The testes from non-castrates were also weighed and studied histologically. Observations were made on the presence or absence of a plug in the urethra representing coagulated secretion from the glands.

Chemical methods

For the chemical analyses of the coagulating gland and the lateral and dorsal prostates, it was necessary to pool tissue from several animals. The seminal vesicle tissue was pooled only in very young males. No analyses were made on the glands at birth. In some cases in the seminal vesicle, secretion and epithelium were analyzed separately. The secretion was obtained by pressing the gland gently, so as to avoid damage to the epithelium, and the gland minus secretion was then cut longitudinally and washed in several changes of 0.9% sodium chloride solution before it was analyzed. The material was homogenized immediately in at least 9 volumes of cold 5% trichloracetic acid, and insoluble matter was removed by centrifugation.

Citric acid was determined by a modification of the method of Speck, Moulder and Evans (15). The lower limit of accurate detection of citric acid was 20 μg. under the conditions employed, but in cases where more than 100 mg. of fresh tissue were available, values for citric acid of less than 20 mg. per 100 gm. were detectable. Fructose was determined by the cystein-carbazole reaction (16). The differences between the optical densities at 560 mμ and 750 mμ were used to calculate the amount of fructose present.

[4] Grateful acknowledgement is made to Dr. Edward Henderson, of the Schering Corporation, Bloomfield, New Jersey, for his generous supply of this hormone.



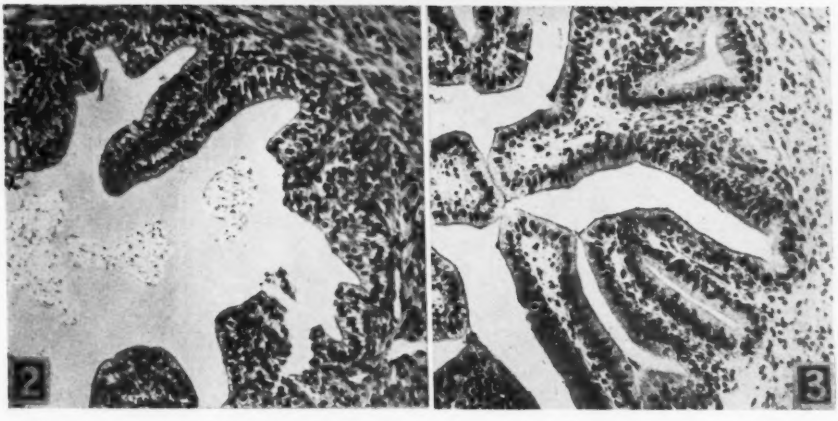


FIG. 1. Accessory reproductive glands of the adult male guinea pig. Insert shows dorsal view.

FIG. 2. Seminal vesicle of normal male at birth. ×150.

FIG. 3. Seminal vesicle of normal male at 10 days of age. Note the greater degree of development than at birth. ×150.

TABLE 1. NORMAL GROWTH AND DEVELOPMENT OF SECRETORY FUNCTION IN THE ACCESSORY REPRODUCTIVE GLANDS OF THE MALE GUINEA PIG

Age (days)	No. of males	Body weight* (gm.)	Organ weights* (mg./100 gm. body weight)				Fructose* (mg./100 gm.)	Citric acid* (mg./100 gm.)			
			Seminal vesicle	Coagulating gland	Lateral prostate	Dorsal prostate	Seminal vesicle	Seminal vesicle	Coagulating gland	Lateral prostate	Dorsal prostate
Birth	11	76 ± 22	69 ± 8 (59–82)	7 ± 2 (4–10)	10 ± 3 (6–15)	29 ± 3 (20–34)					
10	11	178 ± 26	47 ± 11 (32–76)	9 ± 4 (3–16)	14 ± 3 (8–17)	30 ± 6 (20–46)	10.5 ± 3.9 (5.4–16.8)	<20	<20	<20	<20
24	5	224 ± 22	61 ± 20 (31–85)	15 ± 3 (10–17)	16 ± 3 (10–18)	33 ± 8 (23–46)	23.3 (16.2; 30.4)	<20	<20	<20	<20
28	7	269 ± 32	179 ± 79 (98–293)	22 ± 7 (14–36)	22 ± 1 (21–24)	49 ± 7 (42–60)	54.5 ± 34.4 (13.4–101)	98 ± 78 (13–210)	11 (6; 16)	61 (51; 71)	53 (50; 55)
40	6	301 ± 35	171 ± 65 (96–237)	13 ± 3 (10–18)	21 ± 6 (15–31)	59 ± 18 (45–85)	70.2 ± 20.9 (51.2–104)	168 ± 51 (98–260)	30	89	69
60	5	453 ± 43	425 ± 228 (161–733)	34 ± 9 (25–46)	20 ± 8 (14–32)	47 ± 15 (24–61)	59.3 ± 5.2 (54.7–67.6)	216 ± 56 (135–277)	28	210	119
90	5	611 ± 79	443 ± 59 (365–520)	26 ± 4 (21–31)	19 ± 3 (16–23)	58 ± 16 (47–86)	73.1 ± 8.8 (60.0–79.1)	153 ± 67 (56–202)	36	119	72 ± 16 (56–94)
120	9	636 ± 135	502 ± 232 (296–956)	40 ± 20 (22–69)	15 ± 5 (9–21)	53 ± 14 (28–70)	62.7 ± 15.2 (40.4–84.8)	191 ± 33 (124–238)	60 ± 17 (47–85)	108 ± 24 (93–136)	112 ± 30 (73–146)
150	6	779 ± 55	720 ± 417 (298–1,448)	37 ± 5 (31–44)	16 ± 5 (10–23)	75 ± 23 (48–118)	67.6 ± 12.7 (48.5–80.5)	170 ± 25 (133–189)	29 (28; 29)	168 (143; 193)	118 ± 37 (84–130)
180	5	742 ± 106	519 ± 50 (461–558)	31 ± 3 (27–34	20 ± 4 (15–23)	54 ± 6 (40–67)	71.7 ± 13.8 (59.5–95.0)	216 ± 41 (176–266)	46 (40; 53)	164 (163; 165)	92 ± 26 (61–127)

* Mean ± standard deviation. Parenthesis below the mean includes the range or the individual values in cases of only 2 readings.

TABLE 2. EFFECT OF CASTRATION AND OF ANDROGEN TREATMENT ON THE ACCESSORY REPRODUCTIVE GLANDS OF THE MALE GUINEA PIG[1]

	Age (days)	No. of males	Body weight* (gm.)	Organ weights* (mg./100 gm. body weight)				Fructose* (mg./100 gm.)	Citric acid* (mg./100 gm.)			
				Seminal vesicle	Coagulating gland	Lateral prostate	Dorsal prostate	Seminal vesicle	Seminal vesicle	Coagulating gland	Lateral prostate	Dorsal prostate
Castrated for: 2 wks.	24	5	206 ± 19	36 ± 5 (28–40)	7 (5–8)	11 ± 2 (9–13)	21 ± 4 (16–27)	12.2	<20	<20	<20	<20
4 wks.	28	7	283 ± 28	25 ± 3 (21–28)	4 (3–5)	9 ± 2 (5–11)	18 ± 5 (11–22)	11.5 (8.2; 14.9)	<20	<20	<20	<20
4 wks.	40	14	291 ± 48	32 ± 6 (24–45)	6 (3–10)	9 ± 3 (6–15)	18 ± 5 (12–27)	6.3	<20	<20	<20	<20
4 wks.	150	11	670 ± 98	195 ± 124 (77–520)	21 ± 5 (15–30)	14 ± 8 (6–33)	42 ± 12 (24–57)	17.4 ± 9.3 (6.9–30.8)	77 ± 37 (13–124)	23 ± 10 (14–33)	24 (21; 29)	48 ± 12 (35–58)
8 wks.	180	10	784 ± 103	82 ± 3 (58–109)	18 ± 3 (12–24)	11 ± 2 (8–13)	31 ± 12 (16–59)	4.3 ± 3.7 (0.4–13.8)	13 ± 7 (4–28)	7 (6; 8)	7 (4; 9)	18 ± 7 (12–26)
Castrated for 4 wks. +1 mg. t. p.	28	7	310 ± 14	189 ± 51 (142–288)	29 ± 3 (23–33)	24 ± 5 (18–29)	64 ± 10 (47–75)	28.7 ± 14.0 (14.5–48.0)	58 ± 32 (37–128)	23 (15; 31)	117 (99; 135)	77 (71–82)
Castrated for 4 wks. +2.5 mg. t. p.	40	7	294 ± 100	179 ± 56 (102–263)	25 ± 5 (19–32)	14 ± 3 (11–20)	49 ± 11 (29–59)	44.8 ± 29.4 (7.9–82.5)	126 ± 82 (25–200)	22†	128 (36; 220)	46 (45; 47)
Castrated for 8 wks. +2.5 mg. t. p.	180	6	746 ± 70	340 ± 38 (291–383)	33 ± 9 (21–45)	25 ± 6 (16–32)	43 ± 5 (34–48)	52.7 ± 17.0 (31.0–76.8)	152 ± 45 (84–223)	21 (20; 22)	89 (88; 90)	63 ± 16 (46–91)
Non-castrates +1 mg. t. p.	10	8	157 ± 32	116 ± 22 (91–167)	23 ± 3 (17–26)	22 ± 2 (19–24)	53 ± 12 (37–73)	5.7 (3.0–8.2)	16 (<20–37)	<20	<20	26 (<20; 51)
Non-castrates +1 mg. t. p.	28	7	278 ± 69	240 ± 32 (187–358)	37 ± 4 (32–44)	32 ± 6 (25–43)	82 ± 23 (63–122)	28.8 ± 20.3 (11.9–61.0)	81 ± 60 (30–159)	16 (15; 17)	128 (126; 129)	85 (81; 89)

[1] Guinea pigs were castrated at birth, 10 days or 120 days of age. When testosterone propionate (t. p.) was given, the daily dose of 1 mg. was injected from birth to the time of autopsy, and that of 2.5 mg. only during the 2 weeks prior to autopsy.

* Mean ± standard deviation. Parenthesis below the mean includes the range or the individual values in cases of only 2 readings.

† Pooled tissue from 4 males only.

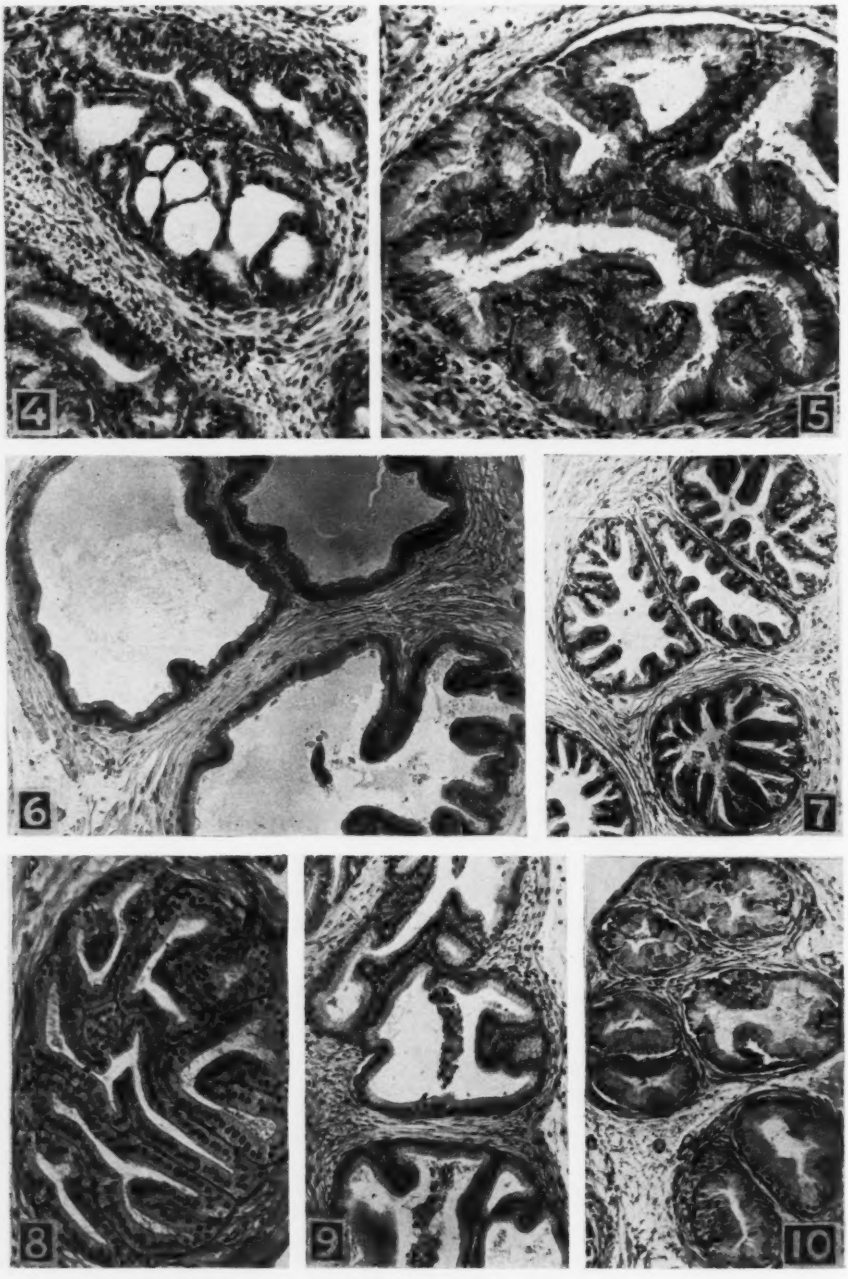


FIG. 4. Dorsal prostate gland at birth. ×150.

FIG. 5. Dorsal prostate of 28-day-old male castrated at birth. Compare with fig. 4 and note considerable histological differentiation in the absence of the testis. ×150.

Optical densities were usually measured within 2 hours after the addition of all the reagents. The ketose sugar in the seminal vesicle gave an absorption spectrum over the range of 45 mμ to 750 mμ, which was identical with that of pure D-fructose. The fructose concentrations determined by the cystein-carbazole reaction agreed in one series within 3% of those found by the method of Roe (17). Although as little as 4 μg. of fructose could be determined by the method used, interference by chromogens formed by the action of concentrated sulfuric acid on substances other than fructose in these glands made values of less than 10 mg./100 gm. of fructose of doubtful significance.

The following experiment demonstrated that most of the fructose present in the seminal vesicle secretion was in the free (non-phosphorylated) form. Fresh secretion expressed from the seminal vesicle of an adult was immediately homogenized in 10 volumes of cold 5% trichloracetic acid. The supernatant fluid after centrifugation contained 75 μg. fructose/cc. An aliquot was made alkaline to phenolphthalein with NaOH, excess barium acetate and 4 volumes of ethanol were added, together with sufficient base to ensure that the mixture was alkaline. After standing for 2 hours at 2° C the mixture was centrifuged. Excess barium was removed from the supernatant fluid with H_2SO_4. As much as 89% of the fructose present in the original aliquot was recovered in this barium-and-alcohol-soluble fraction.

RESULTS

A. *Normal development*

At birth the accessory glands were small and undeveloped (Figs. 2 and 4), but during the first 10 days their absolute weight increased rapidly in proportion to the rapid rate of body growth (Table 1). During this period, there was definite development in the histological structure of all the glands. At 10 days, the seminal vesicle (Fig. 3) had a wider lumen with longer villous projections; the epithelium was pseudostratified in some regions but simple columnar in others and the cells had more free cytoplasm than at birth. There was also secretion in the lumen at 10 days. The acini of the lateral and dorsal prostates were more numerous and larger than at birth and were lined with simple columnar epithelium which also seemed to be secreting material into the lumen (Fig. 9). The coagulating gland had larger acini with lumina just forming at 10 days. By 28 days, correlated with rapid testicular development, the accessory glands had undergone marked growth and differentiation. They had larger acini filled with secretion and lined with simple columnar epithelium, and they

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FIG. 6. Dorsal prostate of adult male. ×100.

FIG. 7. Dorsal prostate of adult male castrated for 8 weeks. A few small acini are shown, with tall epithelial cells; the larger acini did not retain a high epithelium. ×100.

FIG. 8. Coagulating gland of adult male castrated for 8 weeks. One acinus only is shown, but its appearance is typical of the whole gland, which although not distended, contains some secretion and has unregressed epithelium. ×150.

FIG. 9. Lateral prostate of normal 10-day-old male. Note greater degree of development than at birth. ×100.

FIG. 10. Lateral prostate of 40-day-old male castrated at 10 days of age. As in the dorsal prostate, the retention of high epithelium is present throughout; the glands appear like those of the male castrated at birth. ×100.

appeared similar to those of the adult (Fig. 6). After this age, further weight increase in most of the glands was more or less in proportion to body growth, but in the seminal vesicle a more rapid rate of weight increase continued for some time (Table 1). The histological structure of the lateral prostate was quite similar to that of the dorsal.

Significant amounts of fructose were produced in the seminal vesicles of some animals before the age of 28 days, while citric acid did not appear above base levels in any males younger than that age (Table 1). At 28

TABLE 3. THE EFFECT OF CASTRATION AND OF ANDROGEN TREATMENT ON FRUCTOSE AND CITRIC ACID SECRETION IN PREPUBERAL MALE GUINEA PIGS[1]

Age	Weight (mg.)	Fructose (mg./100 gm.)	Citric acid (mg./100 gm.)			
	Seminal vesicle	Seminal vesicle	Seminal vesicle	Coagulating gland	Lateral prostate	Dorsal prostate
A. Normal males injected 1 mg./day of testosterone propionate beginning at birth:						
10 days:		8.2	27	<20	<20	<20
Mean: 177		4.4	37			
(8 males)		7.0	<20	<20	<20	51
		3.0	<20			
28 days:	811	41.9	141			
	462	13.0	34			
(7 males)	641	14.3	30	15	129	81
	558	13.9	38			
	694	46.0	132			
	522	11.9	30	17	126	89
	666	61.0	159			
Mean:	622	28.8	81	16	128	85
B. Males castrated at birth:						
28 days: Mean: 71		14.9	<20	<20	<20	<20
(7 males)		8.2	<20			
C. Males castrated at birth, injected 1 mg./day of testosterone propionate for 28 days:						
28 days	468	25.1	50			71
	684	37.0	53			
(7 males)	444	15.4	39	31	99	82
	479	14.5	37			
	843	43.5	128			
	564	17.2	37	15	135	77
	608	48.0	57			
Mean:	584	28.7	58	23	117	77
D. Normal males:						
28 days:	523	76.2	150			
	701	75.4	144			
(7 males)	307	13.4	13	7	51	55
	497	71.0	125			
	301	21.0	20			
	784	101.0	210	16	71	51
	225	23.4	23			
Mean:	477	54.5	98	11	61	53

[1] Readings of seminal vesicle tissue represent individual values in all except the 10-day and the 28-day-castrate groups. Readings for the other glands are all from pooled tissue.

days, 4 out of 7 males in the normal group secreted adult levels of seminal vesicle fructose and citric acid (Table 3). In 3 cases the glands had insignificant levels of both substances, although they were completely differentiated and indistinguishable from the others. High readings of citric acid were obtained in the lateral and dorsal prostates and very low values in the coagulating gland. In these glands, analyses were made of pooled tissue, so individual variation was not revealed.

Between 40 and 60 days, adult levels of fructose and citric acid in the glands were reached by all the males. The coagulating gland never secreted very large amounts of citric acid, while the level of this substance was high in all the other glands. No significant amounts of fructose were found in the lateral and dorsal prostates or in the coagulating gland.

In the adult animals in which the seminal vesicle secretion and epithelium were analyzed separately, both citric acid and fructose were found almost entirely in the secretion, with very small amounts in the washed gland (Table 4), as previously reported (9, 10). This indicates that, at least in the seminal vesicle, these substances are readily discharged from the epithelium and stored in the secretion. The normal values in this table also agree, in general, with those of the other investigators. The values are considerably lower in the gland plus secretion than in the secretion alone, which shows the effect produced by the bulk of the tissue in lowering frustose and citric acid concentrations.

TABLE 4. DISTRIBUTION OF FRUCTOSE AND CITRIC ACID (MG./100 GM.) IN THE SEMINAL VESICLE OF THE GUINEA PIG

	Age (days)	No. of males	Gland plus secretion		Secretion		Washed gland	
			Fructose	Citric acid	Fructose	Citric acid	Fructose	Citric acid
Normal:	90	4	73.1± 8.8	153±67	129±23	357±93	28.0±7.3	4
	150	6	67.6±12.7	170±25	101±24	355±16	27.7±6.4	14
	180	5	71.7±13.8	216±41	100±26	320±42	12.1±2.1	15
Castrated for 4 weeks:								
	150	5	9.8± 4.2	47±45	32.0	116	10.5	<20
Castrated +testosterone propionate:								
	180	6	52.7±17.0	152±45	91±28	317±27	10.4±3.2	12

A firm urethral plug of coagulated secretion from the glands was first observed in normal males at the age of 28 days. It was present in 2 males, while the other 5 had a soft coagulum.

B. *Prepuberal castration and androgen effect*

Castration at birth or at 10 days prevented normal growth and secretion of fructose and citric acid (Tables 1, 2, 3). A certain degree of histological differentiation, however, continued after castration at birth. This occurred in the lateral and dorsal prostate and to some extent in the coagulating gland, and by 28 days they were much more developed than at birth (Figs. 4 and 5). Following castration at 10 days, no further histological

differentiation occurred, but the glands did not regress and in many acini a high epithelium was retained (Fig. 10).

Testosterone propionate induced marked growth and differentiation in all the glands of castrates. In every male castrated at birth and injected with 1 mg./day for 28 days, the glands were histologically identical to those of the normal at 28 days of age. The seminal vesicle, then, although completely differentiated in all of the cases at this age, had high levels of fructose and citric acid in only about one-half of the animals (Table 3). Therefore, some males did not respond to the treatment by secretory activity. In addition, the values of these substances were within the range of the normal for that age. A coagulum was observed in the urethra of all the males in this group, but it was of firm consistency in only one animal.

In the 40-day-old males castrated at 10 days, the dose of 2.5 mg./day for the last 2 weeks resulted in about the same degree of growth which occurred in the 28-day-old androgen-treated castrates, and, as in the latter group, the histological structure of the glands was completely normal. Secretion of significant levels of fructose and/or citric acid was now stimulated in most of the animals, and a firm coagulum in the urethra was found in most cases.

Androgen injected into normal young from birth to 10 days (1 mg./day) stimulated marked growth (Tables 1, 2) and precocious histological development in all the glands. After treatment for 28 days, the mean weights of all the glands were larger than the normal but within the range of those of the castrate-injected group, especially when absolute weights are considered. Their histological structure was identical to the normal.

In spite of this striking stimulation of growth and structural development, the androgen produced no precocious secretion of frustose at 10 days. Citric acid was slightly increased above base levels in 2 samples of seminal vesicle tissue (each pooled from 2 males) and was increased to a high level (51 mg./100 gm.) in one sample of dorsal prostate representing 3 males. However, the rest of the tissue contained negligible amounts of both substances (Table 3). One of the seminal vesicles in each sample and all of the dorsal prostates were large and well developed. The males from which these seminal vesicles were obtained had a firm urethral plug.

At 28 days, fructose and citric acid in the seminal vesicles were at high levels in about one-half of the group of non-castrate males injected with androgen. These findings were very similar to those in the normal males which received no androgen and to those in the castrate-injected males (Table 3). In all these groups, each animal had values for both substances which were closely parallel. Citric acid secretion in the other glands was also similar in all 3 groups. There was a firm coagulum in the urethra of all except one of the injected males.

Thus, similar results were obtained with this androgen dose (1 mg./day) in non-castrate and castrate males. In the intact males, the injected andro-

gen arrested testicular development. The testes remained small and undeveloped and had the histological appearance of those of the newborn animal. It seems probable that these gonads were secreting little, if any, male hormone, and for this reason, the levels of circulating androgen in the injected castrates and injected intact males were similar. Consequently, since the accessory glands may have been subjected to equivalent hormone levels, their similar response in the two groups is not surprising.

C. *Adult castration and androgen effect*

Castration in the adult reduced the weight of all the glands with the seminal vesicle most affected and the lateral prostate least. Approximately maximal weight reduction was reached by 4 weeks of castration in all the glands except the seminal vesicle, which showed further weight loss by 8 weeks (Tables 1, 2) when it contained less secretion. There was great reduction in acinous diameter in all the glands; the epithelial cells became lower although not completely regressed in the seminal vesicle and in some of the acini of the lateral and dorsal prostates (Fig. 7). In the coagulating gland almost no regression in epithelial height occurred (Fig. 8). The histological changes were sometimes present by 4 weeks, but there was great individual variation, and in some males the changes were not very marked until 8 weeks.

Fructose and citric acid were greatly reduced by 4 weeks. As in the normal seminal vesicle, fructose and citric acid were found almost entirely in the secretion (Table 4), with the values for the secretion alone higher than for the whole gland. By 8 weeks, these secretory products had disappeared almost completely (Table 2). In one male castrated for 4 weeks, the secretion was still coagulable, but none of the 8-week castrates had coagulable secretion.

Androgen treatment for the last 2 weeks (2.5 mg./day) in the 8-week castrate increased the weights of all the glands to almost normal levels and restored normal histological appearance. It also caused a great increase in fructose and/or citric acid, which in the seminal vesicle approached precastration values (Tables 1, 2), with the substances found entirely in the secretion (Table 4). Citric acid values did not return to normal in the other glands, perhaps because normal volumes of fluid secretion were not yet reestablished. All injected males had a firm urethral plug.

DISCUSSION

These findings indicate that mature functional activity, including citric acid and fructose secretion, is established around the age of 4 weeks, after rapid growth and complete histological differentiation. Other investigators (10) have also found fructose secretion initiated at this age, and Sayles (1), using electric ejaculation methods, obtained "coagulable ejaculates"

in 45% of the guinea pigs at 28 days, the proportion gradually increasing with age.

After prepuberal castration, although a certain degree of histological differentiation takes place in most of the glands, functional development is definitely inhibited. As in the rat (4), the secretion of fructose and citric acid is not initiated in the castrate unless androgen is administrered. In addition, prepuberal castrates have glands with little secretion, which is not coagulable, while in castrates injected with androgen the secretion coagulates readily. Sayles (1) also found some degree of histological differentiation in the glands of prepuberal castrate guinea pigs, in spite of the fact that these castrates never developed the ability to produce ejaculates. It is evident, therefore, that certain functions which are dependent on androgen are not being performed by the glands after castration, even in glands with a considerable degree of epithelial differentiation.

The ability of the glands to secrete fructose and citric acid in response to androgen develops gradually. While a high dose of testosterone propionate (1 mg./day) produced marked stimulation of growth and histological differentiation in very young males, it caused little or no precocious secretion of significant levels of these substances before 4 weeks of age, when high levels appear normally. At this age, the results with androgen injection did not differ from the normal findings—in every one of the groups, whether normal, castrate-injected or non-castrate-injected, the same proportion, about one-half of the males, showed high secretory activity, while in the rest the glands were too immature to secrete. Furthermore, there was complete correlation between the levels of the two substances in each animal. In view of these findings, the results of Levey and Szego (10) who claim inhibition of seminal vesicle fructose by androgen injected into intact males, might be attributed to chance. Since they had only five injected animals at 28 days, the absence of secretion, and of fructose, could indicate that the glands in these males were not yet mature enough to respond by secretory activity and would have produced no secretion even if androgen had not been injected. Therefore, it is not that the injected androgen inhibited secretion, but that it did not initiate it.

In the adult, the findings of regression in weight and histological structure after castration are in general agreement with Sayles (2). Although marked weight reductions occur during the first 4 weeks, there are some animals in which the glands are still heavy and contain much secretion. Also in most cases some or all the acini, though greatly reduced in diameter, are lined with high or moderately high epithelium; but it is doubtful that this epithelium keeps the normal rate of secretion, since the material contained in the lumen becomes very reduced in quantity and eventually disappears. With the electric ejaculation method, the ejaculates of adult males were found to be reduced after one week of castration to one-

half to one-tenth of the normal weight and were absent after 3 months (18). Therefore, the secretion found in castrates, for the mot part, must be retained rather than newly produced.

Fructose and citric acid also disappear, though slowly, so that they are present in some of the 4-week castrates. In the rat and the rabbit, fructose disappears within two weeks after testis removal, and citric acid is lost more gradually (4, 19). While the glands of some guinea pigs contain considerable quantities of these substances for as long as 4 weeks, it is not probable that they are being secreted during this period, inasmuch as prepuberal castration prevents this secretory development. Thus, these readings probably represent residual citric acid and fructose in the retained secretion and not freshly secreted material.

It has also been observed that testis removal results in loss in coagulability of the secretion, which becomes progressively more watery. This was generally true in our experiments, with the exception of one case of coagulation after 4 weeks in a male which had an excessively large volume of seminal vesicle fluid. From previous work, it is known that in the guinea pig, vesiculase disappears gradually after castration (20). With the electric ejaculation method (18) it was shown that, although coagulation occurred in ejaculates during two weeks after castration, it was already "decidedly atypical."

It is then evident that, as in the young castrate, the relatively high glandular epithelium retained for some time in the adult castrate is not secreting fructose and/or citric acid, nor is it producing the substances required for coagulation of secretion. In the hamster (21) histological differentiation in the glands of young castrates, as well as the maintenance of normal epithelial height in those of the adult castrate, are even more marked than in the guinea pig. In these two species, the problem still remains as to whether the unregressed cells of these epithelia are active in some other functional capacity, independent of testis hormone, and in the adult hamster, also independent of other hormones (22).

From the fructose and citric acid secretion, organ weights, some aspects of the histological structure, and the coagulation of secretion, it can be concluded that the accessory glands of the guinea pig are definitely under androgen control. Consequently, castration effects in this species are fundamentally similar to those of the rat.

SUMMARY

Fructose and citric acid were found to be secreted in large quantities in the seminal vesicle of the guinea pig. These substances were readily secreted from the epithelial cells and stored in the lumen. Citric acid was also produced in large amounts in the lateral and dorsal prostates, with lower levels found in the coagulating gland. Secretion of adult levels

of these products occurred first at 4 weeks of age. The glands were completely mature in histological structure by then, but they continued to increase in weight until the age of 4 months.

Prepuberal castration completely inhibited growth and the development of specific secretory activities, but it permitted some small degree of histological differentiation. Androgen treatment in the castrates caused entirely normal development in all respects; in intact males, since it produced physiological castration, it had the same effect as in the castrates. The androgen did not produce precocious secretory function, in spite of its stimulating effect on growth and histological structure.

In the adult, castration stopped these secretory activities in the glands and produced marked regression in weight and in acinous diameter, but reduction in epithelial height was slow, incomplete or absent. After androgen treatment, the glands were normal in all respects.

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NOTES AND COMMENTS

ASSAY OF ANTIDIURETIC SUBSTANCE IN PLASMA OF NORMAL AND DIABETES INSIPIDUS DOGS[1]

Controversial reports regarding the presence and assay of circulating antidiuretic substance in dogs have been noted in the literature. Melville (1), employing a method for the quantitative extraction of antidiuretic substance from dog blood using normal dogs as test animals, found this substance to be a normal constituent. Hare, Hickey, and Hare (2) utilizing diabetes insipidus dogs as test animals were unable to detect any significant circulating antidiuretic substance. Within the past few years, interest in this principle has increased as a result of studies implicating it as a factor in control and release of adrenocorticotrophic hormone (3). Furthermore, Lloyd, *et al.* (4) have reported that blood of short term hypophysectomized rats contains no detectable antidiuretic substance, whereas blood of animals hypophysectomized for a month or more contains appreciable levels. In a later study, Lloyd and Pierog (5) reported that serum of diabetes insipidus rats contained very little antidiuretic activity. In view of the reports regarding the presence and absence of circulating antidiuretic substance in normal dogs and the possibility that diabetes insipidus dogs of long duration may contain detectable antidiuretic substance, the following investigation was made employing a different technique. This technique involved assaying circulating antidiuretic substance in dog plasma utilizing rats as test animals in which the material to be assayed is injected intravenously in small quantities and to determine whether dogs with diabetes insipidus due to stalk-section for a minimal period of 18 months contain any detectable antidiuretic substance.

METHODS

Healthy rats weighing 150–230 gm. and maintained in a temperature controlled room on a diet of dog chow cubes with fresh vegetables once a week were used. Assay of the antidiuretic substance was performed on alcohol anesthetized animals. This method is similar to that described by Jeffers, Livezey and Austin (6), except for the following modifications. To insure more adequate and uniform diuresis on the day of the experiment it was found advantageous to hydrate the animals the previous afternoon with a volume of water given by gavage corresponding to 3% of the rats' weight. Only those animals whose urine flow was a minimum of 4.0 cc. per hour during the control period and showed no presence of blood contamination in the urine were utilized. This obviously necessitated using a larger number of animals. An indwelling 24 gauge 3/4 inch needle was placed in the tail vein at the beginning of the experiment and taped to the tail. One-tenth of a cc. of saline injected into the needle prevented any clotting difficulties. Dogs that were used for determination of circulating antidiuretic substance were not permitted water for 24 hours prior to the experiment. Blood for assay from normal and diabetes insipidus dogs was withdrawn and placed in iced centrifuge tubes. The plasma was immediately separated and injected in 0.65 cc. quantities into the tail vein as quickly as possible. A dose of 20 microunits of vasopressin was used as a standard and given at the beginning of the assay to test the sensitivity of the animal.

RESULTS AND DISCUSSION

The level of circulating antidiuretic substance and daily urine output in normal and diabetes insipidus dogs is shown in Table 1. From this data, it can be seen that in normal dogs the values for circulating antidiuretic substance is well above the antidiuretic activity of 20 microunit equivalents of vasopressin which can be detected with this method. These results indicate that the alcohol anesthetized rat is a satisfactory

Received June 5, 1956.

[1] This investigation was supported by Grant H-1836 (C), United States Public Health Service.

TABLE 1. ANTIDIURETIC ACTIVITY OF PLASMA AND URINE OUTPUT IN NORMAL AND DIABETES INSIPIDUS DOGS

Type of animal	No. of animals	ADS of plasma*	Urine output, cc. per day
Normal (48)†	8	Mean 80 Range 45–105	250–450
Diabetes Insipidus (45)	4	Non-Detectable	1450–5000

* Microunit equivalents of vasopressin.
† Numbers in parentheses indicate number of assays.

and sensitive test animal to assay antidiuretic activity in dog plasma. Employing an indwelling needle in the tail vein enables injections to be made without pain which could otherwise evoke an antidiuretic response. Utilizing this method several rats can be used to assay plasma from the same dog in a single day thus providing more uniform results, also small volumes can be assayed without pretreatment that may alter the reliability of the assay.

In contrast to normal dogs, it can be seen that plasma from diabetes insipidus dogs which have been in this condition for a year and a half did not contain any detectable circulating antidiuretic activity suggesting that the material assayed in normal dogs was a physiological constituent. However, it is conceivable that these animals could have a minimal level of antidiuretic substance that was below the sensitivity of this method. As further evidence of a lack of circulating antidiuretic activity in these dogs a comparison of the daily urine output indicates that if there was any circulating antidiuretic activity it was of an insignificant degree to have any effect on urine output since the operative procedure was performed. Therefore, it appears from these results that the stalk-sectioned dog which has completely recovered from the surgical operation for a considerable period of time does not contain any detectable circulating antidiurettic substance.

SUMMARY

Alcohol anesthetized rats constitute a satisfactory and sensitive test for circulating antidiuretic substance in dog plasma. Normal dog plasma contains circulating antidiuretic activity which ranges from 45 to 105 microunit equivalents of vasopressin with a mean of 80 microunit equivalents per cc. Plasma from diabetes insipidus dogs maintained in this condition for a year and a half does not contain any detectable antidiuretic activity.

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AN EXPLANATION OF SOME APPARENT ANOMALIES IN THE ALTERATION OF EOSINOPHIL LEVELS IN THE GUINEA PIG

The guinea pig has been found atypical in the response of its eosinophil level to agents which stimulate adrenal cortical activity. Samter, Erickson and Kofoed (1) found it sluggish in its reaction to ACTH; relatively large doses of the hormone (5 mg every 6 hours) were needed to reduce the peripheral eosinophils to negligible levels. Schweizer (2, 3) found this species also slow in its reaction to epinephrine, the characteristic eosinopenia (-82%) not occurring until 6 hours after injection. More recently, Schweizer (4) has observed that although an increase in circulating eosinophils occurred after castration of the adult guinea pig, the immature male did not have a higher eosinophil count than the sexually mature male.

These apparent anomalies in the eosinophil picture fall into a consistent pattern, however, if cognizance is taken of the simultaneous alterations in the level of circulating polymorphonuclear leucocytes. Samter *et al.* (1) found a marked increase in these cells after ACTH, and Schweizer found the number increased more than 200% three hours after epinephrine administration (2) and more than 200% higher in the mature male than in males less than 30 days old (4). The fact that the increase in polymorphonuclear leucocytes 3 hours after epinephrine injection was even more marked in the splenectomized than in the normal guinea pig points to the bone marrow as the source of these extra cells (2). Since the number of circulating eosinophils represents a balance between factors mobilizing these cells from extra-vascular sources such as the spleen, peritoneal cavity and bone marrow, and those causing their destruction or sequestration, any agent which releases these cells into the circulation will tend to mask their simultaneous destruction. The data suggest that increases in the level of polymorphonuclear leucocytes in the circulating blood probably reflect the release of eosinophilic as well as neutrophilic granulocytes. On this assumption, alterations in the number of eosinophils in the general granulocyte population rather than the number of eosinophils per mm.^{3} of whole blood would serve as a suitable measure of eosinopenia. When the release of granulocytes from the bone marrow is not being enhanced either method of reporting eosinophils will give a reliable picture but with agents like epinephrine and ACTH which mobilize large numbers of polymorphonuclear leucocytes the eosinopenic effect may be obscured by the concurrent release of eosinophils.

In Table 1 some data from earlier experiments have been calculated both as change in the number of eosinophils per mm.^{3} of whole blood, and as change in the number of eosinophils per thousand polymorphonuclear leucocytes. After mild stress, which does not alter the number of polymorphonuclear leucocytes, the percent change is the same whichever method of calculation is used, but the eosinopenic effect of epinephrine three hours after administration is only revealed when the eosinophils are calculated in proportion to the number of polymorphonuclear leucocytes.

The results of some preliminary experiments to determine the best dosages of ACTH for the guinea pig are also summarized in Table 1. Two different preparations, (Preparation I, in gelatin; Preparation II, in aqueous solution) gave essentially the same results whether 1 I.U. or 0.5 I.U./100 gm. body weight was used, but the eosinopenia was more striking and more consistent when eosinophils were calculated per thousand polymorphonuclear leucocytes.

When this method of calculation was used, the discrepancy between the effects of castration and gonadal maturation in the male guinea pig also disappeared (4).

The fact that in other species, despite increase in polymorphonuclear leucocytes, the number of eosinophils per mm.^{3} of blood has given clear evidence of eosinopenia within

Received June 19, 1956.

TABLE 1

Experimental procedure		No. of animals	Eosinophils cells/mm.[3]	Change %	Eosinophils/1000 polymorphonuclear leucocytes	Change %
			Mild Stress (Handling)			
Normal[1]	0 hr.	8	99		26.8	
	1 hr.		70	−29.1%	21.4	−20.0%
	3 hr.		63	−36.2%	16.2	−39.6%
Normal[2]	0 hr.	10	140		32.3	
	6 hr.		134	− 4.3%	28.8	−10.9%
Hypophysectomized[1]	0 hr.	7	262		70.4	
	1 hr.		298	+13.7%	84.2	+19.6%
	0 hr.	7	277		87.4	
	3 hr.		322	+16.2%	99.6	+13.9%
Splenectomized[1]	0 hr.	9	96		17.1	
	1 hr.		97	+ 1.0%	17.7	+ 3.5%
	3 hr.		76	−20.8%	13.9	−18.7%
			Epinephrine			
Normal[1]	0 hr.	9	113		34.3	
	1 hr.		161	+42.4%	49.5	+44.3%
	3 hr.		107	− 5.3%	9.7	−71.7%
Normal[2]	0 hr.	10	108		21.2	
	6 hr.		19	−82.4%	1.5	−92.9%
Hypophysectomized[1]	0 hr.	7	237		68.3	
	1 hr.		306	+29.1%	70.6	+ 3.4%
	0 hr.	7	254		83.8	
	3 hr.		369	+45.2%	68.4	−18.8%
Splenectomized[1]	0 hr.	8	110		23.6	
	1 hr.		124	+12.7%	26.0	+10.1%
	3 hr.		118	− 7.2%	7.2	−69.9%
			Adrenocorticotrophin			
Preparation I[3]	0 hr.	10	132		70.5	
0.5 I.U./100 gm.	4 hr.		74	−43.9%	10.3	−85.4%
body weight	6 hr.		36	−72.7%	6.7	−90.2%
1 I.U./100 gm.	0 hr.	9	206		88.3	
body weight	4 hr.		79	−61.6%	9.3	−89.4%
	6 hr.		70	−66.0%	9.9	−88.7%
Preparation II[4]	0 hr.	9	150		66.2	
0.5 I.U./100 gm.	4 hr.		44	−70.7%	5.2	−92.1%
body weight	6 hr.		32	−78.6%	3.5	−94.7%
1 I.U./100 gm.	0 hr.	9	133		45.3	
body weight	6 hr.		75	−43.6%	7.3	−83.9%

[1] Calculated from data reported elsewhere (2).
[2] Calculated from data reported elsewhere (3).
[3] Kindly supplied by Dr. William Kleinberg, Princeton Laboratories, Princeton, N. J.
[4] Kindly supplied by Dr. W. F. Wenner, The Upjohn Company, Kalamazoo, Michigan.

three hours after administration of epinephrine and ACTH means either that the eosinophil in these species is more sensitive in its response to adrenal cortical hormones or that the extent of mobilization of eosinophils from the bone marrow is not as great as in the guinea pig. On the other hand, Gerstner (5) in a study of chronic effects of various hormones on the blood picture of the rat found that certain inconsistencies in response disappeared when granulocyte-eosinophil ratios were calculated.

SUMMARY

The eosinopenic effects of epinehrine and ACTH in the guinea pig are comparable with those found in other species when the number of eosinophils is calculated per thousand polymorphonuclear leucocytes instead of per mm.^{3} of whole blood. The justification for this method of reporting eosinophils is discussed.

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IN VITRO STUDIES OF THE INFLUENCE OF CORTICOTROPIN, SOMATOTROPIN, THYROTROPIN AND GONADOTROPIN ON ADRENAL STEROIDOGENESIS

A previous investigation (1) demonstrated that intact calf adrenals, perfused with an oxygenated artificial medium, produced and secreted appreciable quantities of corticoids and that corticotropin added to the perfusion fluid substantially enhanced the steroidal output. Little has been published on the direct effect of other adenohypophyseal hormones on adrenal steroidogenesis. The objective of the present study was to ascertain the acute and direct influence on adrenocortical function of somatotropin (STH), thyrotropin (TSH), and gonadotropin (FSH) under the relatively simple and defined experimental conditions previously established.

MATERIALS AND METHODS

Calf adrenals, dissected free of fat and connective tissue and varying from 2.5 to 4.0 gm., were cannulated and perfused via the adrenal vein with an artificial medium of the following composition, in gm./L.: glucose 2.00, NaCl 8.2, KCl 0.25, $CaCl_2$ 0.20, $MgSO_4$ 0.13, $NaHCO_3$ 0.60, NaH_2PO_4 0.05. The perfusion fluid was treated with a continuous stream of water-washed 95% O_2/5% CO_2 and was supplemented with penicillin G (100,000 U./L.) and streptomycin (0.1 gm./L.). The details of the perfusion technique have been presented elsewhere (1).

Experimental groups, consisting of 2 left and 2 right glands, were perfused for 2 hours at 37.5° C in a multicycle system with a liter of medium containing Armour's purified STH[1],

Received July 3, 1956.

[1] Beef somatotropin (STH, Armour lot #285–183) and beef thyrotropin (TSH, Armour lot #317–51) were kindly contributed by Dr. S. L. Steelman, Biochemical Research Dept., The Armour Laboratories.

TSH[1], FSH[2] or ACTH[2]. The control groups, comprising the 4 contralateral organs and differing in weight by less than 4%, were concurrently perfused in another apparatus without these hormonal substances. However, in some studies with STH and TSH, an amount of ACTH equivalent to their "contaminant content" (as bioassayed by adrenal ascorbic acid depletion) was added to the control media.

At the termination of each experiment, the effluents were gassed with nitrogen and immediately frozen in a dry ice-acetone mixture. They were kept in this state until extracted for corticoids with ethyl acetate. The steroid residues were fractionated by paper partition chromatography, and the eluates of the various zones assayed by the Porter-Silber 17α-OH corticosterone and cortisone) and phosphomolybdic acid (corticosterone) reactions. The extraction, chromatography and assay procedures have already been presented in detail (2, 3).

TABLE 1. INFLUENCE OF ACTH, STH, TSH AND FSH ON THE CORTICOID OUTPUT OF ISOLATED PERFUSED CALF ADRENALS

Series	No. of expts.	Treatment of contralateral sets of glands (conc./L.)	ACTH conc. (I.U./L.)	Mean corticoid output[1] (μg.)			Compounds F, E and B	
				Cortisol (F)	Cortisone (E)	Corticosterone (B)	Amount (μg.)	Change[2] (%)
I	4	0.33 I.U. ACTH 0	0.33 0	302 136	117 76	330 235	749 447	+68 ± 14
II	5	1.00 I.U. ACTH 0	1.00 0	500 177	155 85	361 201	1016 463	+120 ± 23
III	4	100 mg. STH[3] 0	1.00 0	404 184	238 145	362 205	1004 534	+ 88 ± 44
IV	4	100 mg. STH[3] 1.00 I.U. ACTH	1.00 1.00	522 619	184 213	380 450	1086 1282	− 15 ± 12
V	4	16.6 mg. TSH[4] 0.33 I.U. ACTH	0.33 0.33	382 374	103 104	223 235	708 713	− 1 ± 8
VI	4	50 Armour U. FSH 0	0 0	237 237	119 128	311 287	667 652	+ 2 ± 11

[1] Micrograms of the quantitatively major corticosteroids secreted, viz., cortisol (F), cortisone (E), and corticosterone (B).

[2] The mean percentage increase (+) or decrease (−) relative to the control output, and the standard deviation.

[3] Activity 75% Armour Standard 22 KR2, prepared by a modified Wilhelmi alcohol procedure and containing 0.01 I.U./mg. ACTH.

[4] 1.2 U.S.P. U./mg. thyrotropin, containing 0.02 I.U./mg. ACTH.

RESULTS AND DISCUSSION

Series I and II of the table indicate that intact calf adrenals perfused *in vitro* respond to corticotropin with an appreciable increment in the production and release of corticoids, and that this response is dose-dependent, being significantly greater at a level of 1.00 than at 0.33 I.U./L. ACTH ($P = 0.01$). Moreover, it is noteworthy that ACTH also induced a qualitative change in the pattern of secretion. Whereas the corticosterone outputs increased 40 and 80%, respectively, the corresponding increments in 17α-OH corticosterone (cortisol), were 122 and 183%, respectively.

In contrast, Series III–VI show that beef somatotropin (100 mg./L.), thyrotropin (16.6 mg./L.) and pituitary gonadotropin (FSH, 50 Armour U./L.) each failed to exhibit an acute effect on adrenocortical activity if compared with an amount of ACTH equivalent to their "contaminant content." It may be noted that they also exerted no significant influence on the pattern of corticoid biogenesis and secretion. Not shown in the table are the data derived from perfusion of contralateral groups of adrenals with 1.0 and 10.0 I.U./L. ACTH; the output was significantly greater at the higher concentration ($P = <0.01$). Inasmuch as 1.0 I.U. ACTH does not induce maximal steroidogenesis, a stimulative effect by 100 mg. STH would probably not be obscured by its ACTH content.

Until definitive means of identifying the presumed ACTH contamination of STH and TSH are available and/or their purity and composition have been established, the

[2] Pituitary gonadotropin (FSH, Armour lot #R377200) and corticotropin (ACTH, Armour's *ACTHAR*, lot #N35307).

possibility exists that these factors possess some inherent adrenocorticotropic activity. Moreover, it should be emphasized that the present negative adrenocorticotropic findings *vis-à-vis* STH, TSH and FSH refer only to their direct and acture action on steroidogenesis and not to their possible *in vivo* ability to induce acute or chronic affects on adrenocortical function, which may or may not be tropic or direct.

SUMMARY

Studies of the acute and direct effect of purified beef anterior pituitary hormones on the steroidogenic function of perfused calf adrenals demonstrated that STH, TSH and FSH, in contrast to ACTH, influenced neither the total output nor pattern of secretion of corticoids if the stimulative effect of their presumed ACTH content is discounted.

Acknowledgment

We are indebted to H. B. Bond for his valuable technical assistance.

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[3] The opinions or assertions contained herein are the private ones of the author and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

ESTROGEN METABOLITES IN HUMAN SERUM[1,2]

The nature of estrogen conjugation and protein binding in human sera has not yet been clearly defined. The relatively low blood titers, even in pregnancy, add considerable complexity to the extraction and characterization of these substances. The problems involved and the areas of disagreement among investigators active in this field have recently been reviewed (1).

The present report concerns the separation and characterization of fluorogenic phenolic metabolites from the serum of a patient during an intravenous infusion of potassium estrone sulfate. An attempt has been made to define in this specific case the nature of the serum estrogens as judged by their behavior when subjected to dialysis, organic solvent extraction and treatment with acid. It was also possible in this instance to employ human serum for an *in vivo* study of estrone metabolism.

MATERIALS AND METHODS

Five cc. of serum were obtained from a patient 18 hours after the start of a 48-hour intravenous infusion of potassium estrone sulfate[3] (50 mg. per hour in 5% glucose).

The sample was bioassayed and frozen for storage until processed. The extraction procedure is outlined in Figure 1. The serum in a Visking membrane was dialyzed against 60 volumes of distilled water for 72 hours. The dialysate was extracted with ether and partitioned between toluene and N/1 NaOH. The phenolic, ether-soluble fraction (I) was assayed

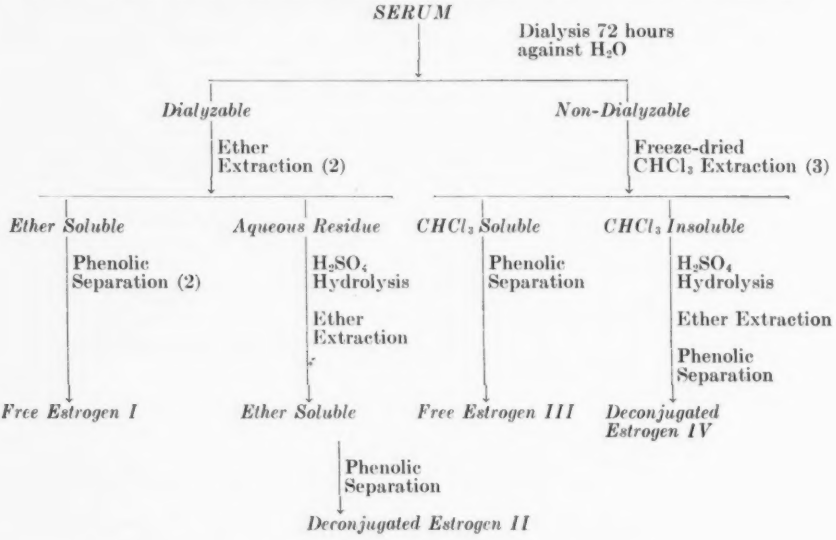
Received March 20, 1956.

[1] This work was supported in part by a grant from the National Cancer Institute, U. S. Public Health Service, and in part by an Institutional Grant from the American Cancer Society, Inc. to the Massachusetts General Hospital.

[2] This is publication #865 of the Cancer Commission of Harvard University.

[3] We are indebted to Dr. Roy Hertz for supplying the sera used in this study.

FIG. 1. FLOW SHEET FOR THE ISOLATION OF ESTROGEN FRACTIONS FROM SERUM



fluorometrically (2). Conjugated dialyzable estrogen was cleaved by treatment with acid, and a phenolic, lipide soluble fraction (II) was isolated and assayed. The non-dialyzable material was dried from the frozen state and the solid residue extracted with chloroform. A phenolic fraction (III) was isolated from the chloroform soluble material. The chloroform insoluble residue was boiled with $1.5N H_2SO_4$, extracted with ether, and a phenolic fraction (IV) prepared from the ether-soluble material. Fractions II and IV were subjected to 24-transfer distribution in the 25-tube steel countercurrent distribution apparatus (4).

The metabolites were characterized by their partition coefficients and their homogeneity established by the agreement of the experimental with the theoretical distribution curves (2) (Fig. 2).

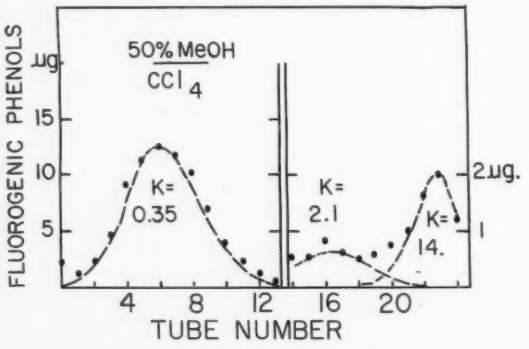


FIG. 2. Counter-current distribution of Fraction IV in a 50% methanol / carbon tetrachloride system. K is the partition coefficient. Experimentally determined values are plotted as black dots; theoretical distribution curves are plotted as dashed lines. The three curves from left to right represent the distribution pattern to be expected from estrone, estradiol and estriol respectively (2).

TABLE 1. ESTROGEN ASSAYS ON HUMAN SERUM FRACTIONS

		Total fluorescence		Counter-current fractions, μg.		
		μg.	%	Estrone	Estradiol	Estriol
Free-Dialyzable	I	5	3			
Conjugated Dialyzable	II	28	17	15.4	0	0.5
Free-Non-Dialyzable	III	17	10			
Conjugated-Non-Dialyzable	IV	115	70	68.5	3.6	6.1
Total		165	100	83.9	3.6	6.6

RESULTS

In control experiments, estrone in aqueous media and estriol in aqueous media and sera were found to pass quantitatively through a Visking membrane into distilled water within 72 hours (5). Analysis of the human serum is summarized in Table 1. The total recovered fluorogenic material was equivalent to 165 ug. of estrone, of which 80% was nondialyzable and could not be extracted with organic solvents prior to hydrolysis. This suggests that a large proportion of the estrogen was protein-bound and conjugated. Intact estrone sulfate probably contributed to the high levels of estrone in the conjugated fractions (II and IV). After countercurrent distribution of the conjugated non-dialyzable fraction (IV), fluorogenic phenols with the partition characteristics of estrone, estradiol and estriol were recognized. Lack of material precluded more definitive identification of the estradiol and estriol.

SUMMARY

An approach has been described for the isolation and characterization of estrogen metabolites in human serum. The presence of fluorogenic phenols with the characteristics of estrone, estradiol and estriol was demonstrated in the serum of a patient receiving an intravenous infusion of potassium estrone sulfate.

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[4] This paper is abstracted from a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Medicine with Honors in Biological Chemistry, 1952.

THE ENDOCRINE SOCIETY

1957 Annual Meeting

The Thirty-ninth Annual Meeting of The Endocrine Society will be held in the Hotel New Yorker, New York, New York, Thursday, Friday, and Saturday, May 30, 31, June 1, 1957.

The Committee on Local Arrangements is Dr. Rulon W. Rawson as Chairman with Drs. Earl T. Engle, Joseph W. Jailer, Warren O. Nelson, and Martin Sonenberg as members of the Committee.

All Scientific Sessions will be held in the Hotel New Yorker. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The Annual Dinner is scheduled for Friday, May 31, at 7:30 p.m., preceded by cocktails at 6:30 p.m.

All members are urged to make hotel reservations immediately inasmuch as the hotels expect to be filled to capacity. Make reservations directly with the New Yorker, advising time of arrival and departure date. Make your reservations now and avoid disappointment.

Those wishing to present papers, which will be strictly limited to ten minutes, should send four copies of the title and abstract to the Vice-President, Dr. Eleanor Venning, Royal Victoria Hospital, Montreal, Quebec, Canada, not later than February 1, 1957. *It is imperative that the abstracts be informative and complete with results and conclusions—not a statement that these will be presented at the meeting*—in order that they may be of reference value and suitable for printing in the program and Journals of the Society. The following regulations for the preparation of abstracts and titles must be carefully followed to insure consideration of the paper for the program:

1. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes or acknowledgments to sponsors can be published. Reference, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formulas cannot be used.
2. The title heading must be arranged as follows:
   - Line 1. Title, not to exceed fifteen words.
   - Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are introduced, i.e., who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, e.g., M.D., of each author should be given after his name.
   - Line 3. Institution of origin and city in which institution is located.

3. The body of the abstract, typed double-space, should follow the heading. The original copy should be on bond paper. There should be three copies.
4. Abstracts should be letter perfect, since there will be no opportunity for proof reading by authors.

1957 AWARDS

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

Medal of The Endocrine Society

In 1954 the Council of the Society voted to establish a medal and an honorarium of $1,000 to be given to an individual for work of special distinction in endocrinology. The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. Dr. Carl R. Moore was the recipient in 1955 and Dr. Frederick L. Hisaw in 1956.

The Ciba Award

The Ciba Award, to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945—Dr. Jane A. Russell; 1946—Dr. Martin M. Hoffman; 1947—Dr. Choh Hao Li; 1948—Dr. Carl Heller; 1949—Dr. George Sayers; 1950—Dr. Oscar M. Hechter; 1951—Dr. Albert Segaloff; 1952—Dr. Seymour Lieberman; 1953—Dr. Sidney Roberts and Dr. Clara M. Szego (Mrs. Roberts); 1954—Dr. I. N. Rosenberg; 1955—Dr. Jack Gross; 1956—Dr. Alfred M. Bongiovanni. Prior to 1952 the Award was $1,200. It is now $1,800. If within twenty-four months of the date of the award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, it will be increased to $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts

in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Schering and The Upjohn Scholars of The Endocrine Society

The Council of The Endocrine Society has established a category of Scholars. These grants have been made available through the generosity of the Schering Corporation and the Upjohn Company, and will be awarded to established investigators and teachers in the field of endocrinology who wish to extend their opportunities for work either in this country or abroad.

The award will not exceed $2,500 annually for each individual and shall be granted on the basis of proposals submitted by the applicant. Such applications should include the estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Medal of the Endocrine Society; the Ciba Award; the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than *November 1* each year.

Proposals for appointment as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by *November 1, 1956.*

The Awards Committee will meet in November and notice of awards to successful nominees and applicants will be made not later than December 1, 1956.